Cytogenetic, phyto-pharmacognostic and bioactivity screening of selected species of *Strobilanthes* Blume (Acanthaceae)

Thesis
submitted to the University of Calicut
for the award of the degree of

DOCTOR OF PHILOSOPHY IN BOTANY

by

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CERTIFICATE

This is to certify that the thesis entitled " **Cytogenetic, phyto-pharmacognostic and bioactivity screening of selected species of *Strobilanthes* Blume (Acanthaceae)**" submitted to the University of Calicut, for the award of the degree of DOCTOR OF PHILOSOPHY IN BOTANY is a record of original research work done by **Reshmi C.,** during the period of study (2017-2022) at the Cell and Molecular Biology Division, Department of Botany, University of Calicut under my supervision and guidance and that it has not formed the basis for award of any degree or diploma. Also certified that the contents in the thesis is subjected to plagiarism check using the software URKUND, and that no text or data is reproduced from other works.

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DECLARATION

I, Reshmi C., hereby declare that the thesis entitled **"Cytogenetic, phyto-pharmacognostic and bioactivity screening of selected species of *Strobilanthes* Blume (Acanthaceae)"** submitted to the University of Calicut, for the award of the degree of DOCTOR OF PHILOSOPHY IN BOTANY is a record of original research work done by me under the supervision and guidance of Dr John E. Thoppil, Professor, Cell and Molecular Biology Division, Department of Botany, University of Calicut and that it has not formed the basis for the award of any degree/diploma to any candidate of any University.

Date: 01/07/2022

RESHMI C.

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I am dedicating my thesis to both my ammas, without whom I will be nothing.

Reshmi C.

CONTENTS

ABBREVIATIONS

°C	-	Degree celsius
%	-	Percentage
µg	-	Microgram
µg/mL	-	Microgram/milliliter
µL	-	Microliter
µM	-	Micromolar
µm	-	Micrometer
A	-	Degree of asymmetry of karyotypes
AIDS	-	Acquired immunodeficiency syndrome
A1	-	Intrachromosomal asymmetry index
A2	-	Interchromosomal asymmetry index
$AlCl_3$	-	Aluminium chloride
As K%	-	Karyotype asymmetry index
AO	-	Acridine Orange
ATP	-	Adenosine triphosphate
Bcl-2	-	B-cell lymphoma 2
Caspase 3	-	Cysteine-aspartic proteases 3
CAT	-	Catalase
cDNA	-	Complementary DNA
Cd	-	Cadmium
CE	-	Caffeine equivalent
COX	-	Cyclooxygenase
CO_2	-	Carbon dioxide
DI	-	Dispersion index

DLD1	-	Human colorectal adenocarcinoma
DMEM	-	Dulbecco's Modified Eagle’s Medium
DMSO	-	Dimethylsulphoxide
DPPH	-	2,2-diphenyl-1-picrylhydrazyl
EDTA	-	Ethylene diamine tetra acetic acid
EDX	-	Energy dispersive X- ray spectroscopy
EtBr	-	Ethidium Bromide
eV	-	Electron volt
$FeCl_3$	-	Ferric chloride
g	-	Gram
g DW	-	Gram dry weight
GAE	-	Gallic acid equivalent
GC	-	Gas Chromatography
GC-MS	-	Gas Chromatography-Mass Spectrometry
h	-	Hour
HNO	-	Nitric acid
H_2O_2	-	Hydrogen peroxide
H_2SO_4	-	Sulphuric acid
HCl	-	Hydrochloric acid
HR-LC/MS	-	High Resolution Liquid Chromatography/Mass Spectrometry
IC_{50}	-	Inhibitory concentration 50%
ICP-MS	-	Inductively coupled plasma mass spectrometry
IPP	-	Isopentenyl pyrophosphate
KOH	-	Potassium hydroxide
L	-	Liter

L929	-	Normal fibroblast cell line
L/min	-	Liter per minute
LC	-	Liquid Chromatography
LD_{50}	-	Least Dose 50%
LDL	-	Low density lipoproteins
LE	-	Linalool equivalent
M	-	Molar
m/z	-	Mass to charge ratio
mg	-	Milligram
min	-	Minutes
mL	-	Milliliter
mM	-	Millimolar
Mn	-	Manganese
MS	-	Mass spectrum
MS Q-TOF	-	Mass spectrometry quadrupole time of flight
MTT	-	3-(4,5-Dimethylthiazol-2-Yl)-2,5-diphenyltetrazolium bromide
N	-	Normal
Na_2CO_3	-	Sodium carbonate
NADH	-	Nicotinamide adenine dinucleotide hydrogen
$NaNO_2$	-	Sodium nitrate
NaOH	-	Sodium hydroxide
NBT	-	Nitro blue tetrazolium
Ni	-	Nickel
nm	-	Nanometer
nsm (-)	-	Nearly sub-median

OD	-	Optical density
Pb	-	Lead
PBS	-	Phosphate buffered saline
PCR	-	Polymerase chain reaction
PDB	-	Paradichlorobenzene
QE	-	Quercetin equivalent
ROS	-	Reactive oxygen species
rpm	-	Revolutions per minute
RCL	-	Range of chromosome length
RT- qPCR	-	Quantitative reverse transcription polymerase chain reaction
SE	-	Standard error
SEM	-	Scanning electron microscope
SOD	-	Superoxide dismutase
TBA	-	Thiobarbituric acid
TCA	-	Trichloroacetic acid
TCL	-	Total chromosome length
TE	-	Tris EDTA
TF%	-	Total forma percentage
TRXs	-	Thioredoxins
UV	-	Ultraviolet
V	-	Volt
w/v	-	Weight per volume
WHO	-	World Health Organization

INTRODUCTION

A plethora of records regarding the use of plants to treat various diseases are available since the beginning of human civilization. The early great civilizations of the ancient Chinese, Indians and North Africans have provided written evidence of man's inventiveness in using plants for the treatment of many diseases (Phillipson, 2001). Medicinal plants produce a variety of compounds in which most of them have unknown therapeutic activities. The efficacy of plant-based drugs used in traditional medicine has been paid great attention to, because they are cheap having little side-effects (Dash et al., 2011). The free radicals generated in the body lead to cellular or oxidative stress, which eventually causes diseases like diabetes, cirrhosis, cancer *etc.* (Valko et al., 2007). Plants contain a rich source of free radical scavenging molecules such as phenols, flavonoids, vitamins, terpenoids that hold potential antioxidant properties (Upadhyay et al., 2010). Recent estimates of the World Health Organisation (WHO) suggests that 80% of the world population use traditional medicine and most of this therapy involves the use of plant extracts and their active components (Aiello et al., 2019).

The in-depth relation between human and plants are for various purposes. It includes food, shelter, medicine *etc.* In most of the holy books, the plants were considered as God. The first preference of modern man for therapy was in synthetic and chemical drugs, but the deleterious effects of these are dragging them back to the ancient medicines. The ancient medicines or simply herbal medicines were proved to have permanent curing, which is the main advantage of plant-based medicines over synthetic drugs. The major phytochemical classes in plants include terpenes, phenols, flavonoids, alkaloids, saponins, phlobatannins, coumarins *etc*. These constituents may

synergistically act each other to enhance the bioactivities and pharmacological importance of a plant.

Humans' trust in nature and low side effects makes plants invaluable in medicine since ancient times. The underexplored plants enhance the growing research field, a fruitful and precious one in drug discoveries. The journey in search of medicine has the same age as that of mankind. The first line faith and basic health services for people in remote areas and economically backward ones are been provided by herbal medicines.

Cytological studies, more precisely cytogenetics have given proper ancestral history and interrelationships between species. As per the arguments by Levin (2019) the future plant speciation solely depends on autopolyploids because it is positively correlated with nutrients, humidity, temperature variation and high level of herbivory. The conventional stained chromosomes are the first step for any chromosome study. The preparation at its high quality will be the key feature. According to Lewitsky (1931) who proposed the term ‘karyotype’, it is the external appearance of somatic chromosome complements. Karyotype, karyograms and idiograms are the terms related to traditional cytogenetics. Karyotype elucidation is clearly depending on the size of chromosomes and the position of centromeres. It is an assemblage of types of chromosomes in the chromosome complement.

The German botanist Eduard Adolf Strasburger put forward the term ‘chromosome’ in 1888. Later, the statement made by Strasburger was translated by Sir William B. Benham and it read as ‘I must beg leave to propose a separate technical name ‘chromosome’ for those things which have been called by Boveri ‘chromatic elements’, in which there occurs one of the most important acts in karyokinesis, *viz*. the longitudinal splitting. They are so important that a special and shorter name appears useful. If the term I propose is practically applicable it will become familiar, otherwise it will soon sink

into oblivion' (Zacharias, 2001). The limit of the chromosome size a cell can withstand is, the longest chromosome arm must be less than the average length of the spindle axis at telophase.

Cytological indices have immense importance as a proven tool for supporting other scientific data in explaining the evolutionary scenario of an individual. Karyotype analysis is a reliable procedure for aneuploidy identification and chromosomal rearrangements (Gouas et al., 2008). The morphological characters of the chromosomes are the major cause of genetic abnormalities reported so far (Bickmore, 2001). Karyotyping is an advanced genotyping technique for the visual examination of genetic changes. It can be achieved by the modern techniques in cytogenetics along with the conventional stained karyotypes (Montazerinezhad et al., 2020).

The position of the centromere is the landmark of the chromosome while considering the chromosome morphology. Several classifications were proposed for naming chromosomes. According to Stebbins (1971) the four categories were telocentric, acrocentric, submetacentric and metacentric. They were also classified as median, nearly median, nearly submedian, submedian, nearly subterminal, sub terminal, nearly terminal and terminal (Sharma & Chandhnri, 1964; Bose & Flory, 1965). The Levan et al. system of nomenclature (1964) was based on the difference between long arm, short arm and arm ratio. In this concept, the whole chromosome is taken as 10 units. Adhikary in 1974 recognized 4 fixed points and 4 intermediate regions. This system was based on arm ratios. Later a modification to this was proposed by Abraham and Prasad (1983). It includes 10 positions with 4 fixed points and 6 definite regions. This was proved to be a better system than that proposed by Adhikary in 1963, Huziwara in 1962 and Levan et al. in 1964. According to Vimala et al. (2021) karyotype description includes chromosome number, genome size, the absolute and relative size of the

chromosomes, centromere position leading to the symmetry of chromosomes, number and position of secondary constriction or satellite and heterochromatin distribution.

The phytochemical (Phyto in Greek means plant) profiling gives an account of the potential phytocompounds within a plant which can be further used for future research. Before World War 2 natural products isolated from higher plants became curing agents and a large number of them are still in use today. This series includes quinine from *Cinchona* bark, morphine and codeine from the latex of the opium poppy, digoxin from *Digitalis* leaves, atropine and hyoscine from Solanaceae members (Phillipson, 2001).

Qualitative and quantitative phytochemical profiling has been given significant importance in identifying the potential phytocompounds having remarkable bioactivities. Alkaloids from natural sources play an important role in the ecology of organisms that produces them. The pharmacological, veterinary, and medical uses of alkaloids are of great importance. They are present in plants, beverages, well-cooked foods and even in tobacco smoke (Patel et al., 2012). They are used as anesthetics and central nervous system stimulants (Madziga et al., 2010). Both primary and secondary metabolites should be investigated for the study of a plant drug. The natural chemical composition of a plant can be achieved by various qualitative tests.

Even though phytocompounds are produced for self-defense in plants, they are useful to mankind for various purposes. They accumulate in different parts of the plants, and vary from plant to plant. They protect plant cells from environmental hazards such as pollution, stress, drought, UV exposure, pathogenic conditions *etc*. (Mathi, 2000). The biological properties of these metabolites include antioxidant activity, antimicrobial effect, modulation of detoxification enzymes, stimulation of the immune system, decrease of

platelet aggregation, modulation of hormone metabolism and anticancer activity (Saxena et al., 2013).

Phytoconstituents can be analyzed by various sensitive techniques such as GC/MS, LC/MS, HR-LC/MS *etc*. These methods can be used for detecting biologically potent chemicals within a sample (Zwiener & Frimmel, 2004). The organic matter within a sample is complex because it may contain thousands of individual constituents. The exact composition cannot be analyzed using a single method. Hence a combination of analytical techniques is used. GC/MS analysis reveals the volatile compounds in a system.

The high-resolution liquid chromatography with mass spectroscopy gives the information regarding the molecular structures within a complex of databases (Petras et al., 2017). In a high-resolution full scan with LC/MS, the data obtained gives the fingerprint of all compounds in the system. The advantages include the high MS specificity without reduction in the sensitivity of detection, the ability to identify all compounds in both positive and negative ionization modes, the ability to reprocess, find new metabolites, and prohibit anonymous drugs. Moreover, it can be utilized for testing the metabolic state of a person in sports to analyze the illegal use of performance boosters (Abushareeda et al., 2018). Within the LC/MS lipidomics applications, 80% is based on mass spectrometers that are efficient in full mass spectra acquisition (Cajka & Fiehn, 2014). The resolving power, mass accuracy, isotope-profile error, sensitivity, maximum spectral acquisition speed, linear dynamic range, and availability of tandem-MS function are the basic qualities essential for a high-resolution mass analyzer (Holčapek et al., 2012).

The basic level of documentation in this chromatography technique is the molecular weight of a metabolite using mass spectrometry. The accurate mass is obtained with a proper molecular formula. The separation and

identification of molecules will be based on retention time, database difference (library), experimental mass to charge ratio, metabolite class and proposed compounds (Noumi et al., 2020). Generally, the experimentally measured mass will not be the exact proposed molecular formula, so the formula given by the software has a deviation from the exact mass measured in parts per million.

There are three major types of mass spectrometers used in high-resolution measurements in metabolomics: Time of flight mass spectrometers (TOFs), ion cyclotron resonance mass spectrometers and Orbitrap (Watson, 2013). Among this TOF analyzer is the cheapest high-resolution mass analyzer. It has some remarkable characters such as acquisition speed, m/z range and relatively good resolving power and mass accuracy (Holčapek et al., 2012).

The term pharmacognosy was used first between 1811 and 1815 as knowledge of the drug. Later the concept was restricted to medical substances from the plant, animal or from the natural, crude or unprepared state (Kinghorn, 2001). Proper identification of the botanical source of the drug which exists as a fragmentary material or powder form can be mainly relied on pharmacognosy (Saritha & Brindha, 2001). Microscopy is an important tool in studies and authentication of crude drugs, guidance for future research and for pharmaceutical industries, it is a prominent index (Sethuramani et al., 2021). In herbal therapy quality, efficacy and safety are relevant factors (Padmavathi et al., 2011). To evaluate the quality, safety and efficacy of herbal plants, World Health Organisation (WHO) has many standards The standardisation and quality control of a crude drug can be obtained by microscopic and macroscopic evaluation and chemical profiling (Yadav & Dixit, 2008). The exploitation of similar medicinal plants in drug preparation

can be eradicated using these methods. The use of adulterants increases when there is a scarcity of the original plant or are very costly.

The atomic spectroscopic techniques such as inductively coupled plasma mass spectrometry (ICP-MS), flame atomic absorption (FAA), electrothermal atomization (ETA), and inductively coupled plasma optical emission spectrophotometer (ICP-OES) were used for the elemental analysis of a sample. Among these ICP-MS is the fastest growing trace element technique available today. It is a powerful tool to quantify the concentration of a sample. The concentration of the various elements present in the sample is expressed in parts per billion or parts per million. The liquid sample is pumped with a peristaltic pump into a nebulizer which is converted into a fine aerosol with argon gas. These fine droplets of the aerosol are separated from the large droplets with the help of a spray chamber. Then they are emerged from the exit tube of the spray chamber and are later elated into the plasma torch *via* a sample injector (Thomas, 2008).

Reactive oxygen species (ROS) is considered as a necessary evil for organisms since it has both beneficial and detrimental effects based on its level. ROS or free radicals contain an unstable electron that tends to react with other species. They react with starch, fat, proteins and nucleic acids. The biological system cannot withstand the higher concentrations of free radicals but at its moderate level, they guard against various infections. ROS break down the peptide chains, oxidize amino acids, and thereby disturb the membrane. Hence it damages the tissue which eventually leads to many dreadful diseases such as cancer, neurodegenerative diseases, heart and liver diseases.

Detoxification of ROS is done by various cellular enzymatic and non-enzymatic mechanisms. In many human cancers, there is an increase in ROS stress which eventually causes damage to normal cells. Plants have the

inherent capacity to scavenge these free radicals and thereby protect the cells. The overall capabilities of herbal medicines enhance their way through the potential pharmaceutical drug discovery. The hydroxyl radicals will damage proteins in the living cells and have the capacity to join nucleotides in DNA to cause strand breakage eventually leading to mutagenesis, cytotoxicity, carcinogenesis *etc.* The oxygen radicals may attack DNA either at the sugar or the base and produces many radical products (Sundaram et al., 2021).

The only remedy against ROS is antioxidants. They conserve cellular functions by mollifying ROS. There are various endogenous and exogenous antioxidants. The endogenous are of two types - enzymatic and non-enzymatic. Antioxidants inhibit the formation of new radicals, traps the free radicals to avoid chain reaction and it restores the destruction caused by radicals (Neha et al., 2019). Hence, the oxidative stress that arise due to ROS will be balanced by the antioxidants. Generally, living cells are exposed to ROS throughout their life at a specific level only because they are produced as a by-product of various metabolism or by stress. The internal producers of free radicals are mitochondria, peroxisomes *etc*. Whereas the external factors include smoking, drugs, ozone, pesticides, environmental pollutants *etc.* The various mechanism of free radicals includes reduction, oxidation, hydrogen abstraction, self-annihilation reaction, disproportionation *etc*. (Carocho & Ferreira, 2013).

The antioxidant defending can be enhanced by intaking exogenous antioxidants such as ascorbic acid, α-tocopherol, polyphenols, carotenoids *etc.* (Lourenço et al., 2019). The higher stability, performance, low cost and wide availability make the use of synthetic antioxidants over natural ones. The safety issues regarding the use of synthetic antioxidants are not negligible. So, natural antioxidants are recommended. In the treatment of cancer, antioxidants act beneficially. It increases cancer cell progression

(Sarangarajan et al., 2017). ROS promotes cell migration and invasion in metastasis of cancer cells. Hence the scavenging potential of antioxidants is very crucial in cancer therapies. At the molecular level, the harmful effects of electrophiles or ROS can be refuted by Keap1 and Nrf2 related signaling cascades. Keap1 is a sensor for these oxidative changes whereas Nrf2, in turn helps in the transcription of key antioxidant enzymes. Parallelly thioredoxin reductase 1 regulates Nrf2 to maintain the balance between oxidants and reductants.

The cytotoxic effects of various substances are analyzed *in vivo* and *in vitro*. These are essential in drug discoveries. The adverse effects of drugs must be analysed for a better understanding of the impact on humans. The potential toxicity of a crude drug must be verified by various studies before implementing it as a medicine. *Allium cepa* is a model organism to evaluate the cytotoxic effect of a compound. It is a reliable technique widely used by researchers as a pre-scanning test material. It is accepted as the genetic model to evaluate the genotoxic effects such as chromosome aberrations and disturbances in the mitotic cell cycle. Through this, the assessment of different genetic endpoints such as mitotic index and chromosome aberration was analysed (Yuet Ping et al., 2012).

Among the malignancies detected so far, colon cancer is the most dreadful disease. The alarming increase in colon cancer is due to the modern food habits of humans. Fast food has been an important guest in our dining. But these unhealthy preparations make lazy people and diseased ones at a very young age. The accumulation of toxic substances in the body invites so many other difficulties which may or may not be cured.

The progressive accumulation of mutations in genes leads to uncontrolled somatic cell proliferation which causes cancer. The oncogenes present in normal cells transform them into cancer cells when overexpressed.

When inflammation occurs, the body starts the complicated wound healing. In the wounded area, normal molecular metabolism is interrupted and induces cancerization of some cells. The continuous activation of oncogenes will increase the speed of transformation of normal cells to cancerous ones (Mitsushita et al., 2004). The increased number of cancer patients has led the researchers to focus on new anticancer agents from natural products. More than 60% of anticancer drugs are derived from natural sources (Talib et al., 2020). The different mechanisms against cancer are apoptosis induction, immune system modulation and angiogenesis inhibition (Rayan et al., 2017).

Cell line technology plays a pivotal role in cancer research. The *in vivo* and *in vitro* studies are essential for the drug release towards human beings. The *in vivo* studies are performed within an organism whereas the *in vitro* studies are conducted in laboratory conditions. Several cell lines were used for the *in vivo* studies. The effect of a compound/drug is experimentally investigated in cell lines mainly cancer cell lines. Based on the mode of action or growth inhibition in cancer cell lines, the inhibition percentage of the drug is verified. This is taken forward for future studies *in vitro.*

Since any drug with DNA damaging ability would be expected to alter cell cycle progression, their cell cycle analysis should be considered before addressing it as a remedy (Schafer, 1998). The signaling pathways monitor the cell cycle progression in a regular manner. They are commonly known as cell cycle checkpoints. A cell can arrest the cell cycle checkpoints momentarily for cellular damage to be repaired, dissipation of an exogenous cellular stress signal, or for availability of essential growth factors (Pietenpol & Stewart, 2002). If the signals cannot overcome the repair, the cell will be assigned to programmed cell death. The checkpoints are activated in the G1/S phase or in the G2/M phase to avoid replication of damaged DNA or prevent segregation of damaged chromosomes during mitosis respectively (Evans et

al., 1983). The checkpoints are helpful in accomplishing phase-specific events and help maintain genetic integrity (Hartwell & Weinert, 1989). The phase progression from G2 to M requires the Cdk1/cyclin B kinase complex. It is regulated by either dephosphorylation of Cdk1 or binding of cyclin B and translocates the complex into the nucleus (Herman-Antosiewicz & Singh, 2004). With the help of cell cycle analysis, the genes involved in apoptosis can be found and further studies in gene expression can be analyzed.

The cell cycle is upregulated or downregulated by several genes. The proper maintenance and checking are necessary for the appropriate advancement of a cell cycle. The gene expression studies will be able to make a clear-cut distinction about the crude drug/isolated compound in promoting or inhibiting these genes. Apoptosis promoting genes are necessary for the cell cycle arrest during uncontrolled cell proliferation. Whereas the anti-apoptotic genes in normal cells control the unrestricted division and growth of the cell cycle. The gene families involved and/or collaborate in the process of apoptosis include caspases, inhibitors of apoptosis proteins, B cell lymphoma (Bcl)-2 family of genes, tumor necrosis factor (TNF) receptor gene superfamily, p53 *etc.* (Kiraz et al., 2016). The apoptotic genes include p53, p16, p21, p27, E2F genes, FHIT, PTEN *etc*. The anti-apoptotic genes were Bcl-XL, Bcl-w, Bcl-2a, Mcl1 and the pro-apoptotic genes include Bax, Bak1, Bim, Bid *etc*. If proper apoptosis is not done in unwanted cells, the mutations could continue to accumulate in the cells and eventually lead to diseases (Horvitz, 1994). In mammals 14 different caspases are present and they are categorized as the initiators including caspase-2, -8, -9, and -10; the effectors including caspase-3, -6, -7, and -14 and the cytokine activators including caspase-1, -4, -5, -11, -12, and -13 (Shi, 2002; Chowdhury et al., 2008).

Two different pathways are involved in apoptosis: (i) intrinsic pathway that is *via* release of cytochrome c from the mitochondria, and (ii) extrinsic

pathway when the death receptor is activated by a signal from outside the cell. Later different intermediate molecules are activated by signaling cascade. Finally, both pathways meet up in the final caspase activation leading to cleavage of proteins (Ghobrial et al., 2005). In the intrinsic pathway, cells undergo apoptosis in either a positive or negative manner. The positive stimuli activate all the mediators and negative stimuli eliminate the inhibitor of apoptosis (Saelens et al., 2004). Whereas in extrinsic pathways the signaling is by membrane-bound death receptors belonging to the tumor necrosis factor (TNF) gene superfamily. The primary signal is by the interaction between cell membrane death receptors such as Fas ligand/ FasR, TNF/TNF R1, Apo2L/DR4, or TNF-related apoptosis inducing ligand (TRAIL) R1 (Locksley et al., 2001). The interpretation of the type pathway involved in apoptosis can be concluded from the gene expression analysis.

Strobilanthes genus consists of about 450 species that are delimited to the hills of tropical Asia. About 148 species are represented in India of which 72 are endemic with the diversity occurring in two regions, *viz.* the Eastern Himalayas and the Western Ghats (Bera et al., 2020). It is mostly native to tropical Asia and Madagascar, but with a few species extending north into the temperate regions of Asia (Shende et al., 2015).

Systematic position (APG IV, 2016)

Kingdom : Plantae

Sub-kingdom : Phanerogamae

Division : Angiospermae

Class : Eudicots

Sub-class : Asterids

Order : Lamiales

Family : Acanthaceae

Genus : *Strobilanthes*

Bremekamp (1944) termed the flowering periodicity of *Strobilanthes* as 'plietesials' and Fyson as multiennial. It means they flower once in several years (1-13) and then dies. This nature of flowering sometimes covers the entire hills as in Nilgiris by *S. kunthiana* in every 12 years. This is not true for all species since survival after flowering stage is shown by *S. callosus* as well as in rhizomatous species. In contradicting to their reproductive phase, they sporadically grow vegetatively. The evergreen Shola forests are mainly *Strobilanthes* vegetation. The circumscription of the genus is large with small grassland species and large shola species making canopies (*S. gracilis, S. homotropous*). The rhizomatous species withstand the seasonal forest fire. The generally applied names of the genus were Carvi, Nelu or Kurinji.

The diversity of *Strobilanthes*, its morphology, ecology and life history (Moylan et al., 2004) in peninsular India makes it an evergreen forest. Venu (2006) had reported about 59 species and 2 varieties from there. The name *Strobilanthes* is derived from the Latin words 'Strobilos' meaning cone and 'anthos' meaning flower or shoot. The height of the plant varies from 30 cm to several meters. The scientific description of *Strobilanthes* was first done by Christian Gottfried Daniel Nees von Esenbeck in the 19th century. The unusual flowering behavior in most of the species varies in the flowering cycle. This longer interval of flowering may range from one to twelve years. The secondary metabolites present in Acanthaceae point out its medicinal role and significance to researchers to study the various aspects of the family (Khan et al., 2017).

Acanthaceae is a pantropical family with the important genera coming under being *Acanthus, Andrographis, Barleria, Thunbergia, Ruellia, Justicia, Ecbolium, Strobilanthes etc*. Among these *Strobilanthes* Blume is the second largest genus in the family with comparatively larger habits (Wood, 1995). It grows gregariously, flower and set fruits after a definite period of growth which is termed as pleitesial flowering (Bremekamp, 1944). The genus *Strobilanthes* consists of about 350 species distributed throughout South and Southeast Asia, Malaysia and Northern Australia. Out of the 43 species known from Kerala, 38 are endemic to peninsular India (Sasidharan, 2000).

Despite of the medicinal properties, the economic utility of the genus is not explored. The wood is hard but brittle due to the large pith hence it acts as a good fuel. In villages, *Strobilanthes* stems along with dung are used for constructing walls of houses. Among the species under investigation *S. anamallaica* and *S. virendrakumarana* are endemic species of Southern Western Ghats with plietesial nature of flowering. These two species have no record of bioactivity report. But *S. hamiltoniana* was proven for its few bioactivities (Baby & Raphael, 2018), but lacks reports on *in vivo* studies and chromosome analysis. A comparative approach of these three species with respect to their chromosome morphology, phyto-pharmacognostic characters and bioactivities were focused in this investigation.

The important objectives of the study are as follows:

- Cytogenetical analysis of the three species *viz. Strobilanthes anamallaica, Strobilanthes hamiltoniana* and *Strobilanthes virendrakumarana* - karyotyping and karyomorphological parameters evaluation
- Pharmacognostic analysis - powder microscopic and ICP-MS analysis of all the species

- ❖ Phytochemical characterisation - preliminary qualitative and quantitative analysis, GC/MS analysis and HR-LC/MS analysis
- ❖ Bioactivity screening:
  - ✓ Antioxidant assays - DPPH free radical scavenging assay, hydroxyl free radical assay, superoxide free radical scavenging assay and reducing power assay
  - ✓ Cytotoxicity assay on *Allium cepa* cells treated with the extract for 24 h
  - ✓ Cytotoxicity on DLD1 (human colorectal cancer cells) and L929 cell lines (normal) - MTT assay
  - ✓ Antiproliferative studies on DLD1 cell lines - MTT assay, apoptotic detection using double staining with AO-EtBr
  - ✓ Flow cytometric analysis and gene expression studies on the most potent species

REVIEW OF LITERATURE

I. Medicinal use of *Strobilanthes*

Traditional medicine was purely based on plant and plant products. The vast variety of medicinal plants were explored by our ancestors in ancient times itself where modern technology had not even named them. Each plant has its medicinal property which can be used as a potential drug against diseases. With the help of modern sophisticated technologies, the underexplored and unknown pharmaceutical activities of plants were revealed nowadays. Likewise, the species of *Strobilanthes* were used in traditional medicine from time immemorial, still, the complete picture in respect of the bioactivities and medicinal quality of the genus is not discovered so far. The bioactivities, phytochemicals revealed and the chromosome number reports of the species in the genus so far discovered are discussed here.

In Kerala, some species of *Strobilanthes* were widely used in Ayurveda, especially the therapeutic drug 'Sahachara' which is widely used against rheumatism, neurological disorders, ulcers, glandular swellings, poisonous affections, skin diseases, leprosy and gum diseases (Sivarajan & Balachandran, 1994). Traditional medicine in Malaysia and Indonesia uses filtrates of boiled *S. crispus* leaves as antidiabetic, diuretic, antilytic and laxative (Bakar et al., 2006). The plant is a woody shrub which is locally known in Malaysia as *Pecah kaca, Pecah beling, Karang jin, Bayam karang* and yellow *Strobilanthes* in English (Ghasemzadeh et al., 2015). It is used by villagers in West Java to treat hepatitis and postpartum remedy. In Chinese, it is called *Hei Mian Jiang Jun* which means Black-faced General. Chinese drug '*Shengma'* is a combination of *Serratula* and *Strobilanthes* (Li et al., 1997).

Ban-lan-gen is used to describe root tissues of several indistinguishable plant species in China which are used as medicine. Indigo, indirubin and tryptanthrin are the key compounds of *Ban-len-gen* (Chen et al., 2018). *S. cusia* is one of them which is widely used in the textile industry due to rich indigo. Transcriptomic analysis revealed that the genes encoding the indigo biosynthesis were monooxygenase, uridine diphosphate-glycosyl transferase and β-glucosidase. The genetic information of dye-yielding plants can increase the application of genetic engineering in industries (Xu et al., 2020). *S. bantonensis* is called 'purple Ban-len-gen' due to its similarity in root with *S. cusia.* This herb is used against influenza virus infection in China and Vietnam (Siddique & Saleem, et al., 2011).

Indigo Naturalis is used against skin psoriasis and nail psoriasis. Nail psoriasis includes nail dystrophy, thickening, loss of lustre, raising, colour changes and friability (Chiang et al., 2013). It is a powder extracted from the branches and leaves of indigo-producing plants by fermenting in fresh water for several days of decomposition. Ethyl acetate extract of Indigo Naturalis isolated from *S. formosanus* inhibits gram-positive bacteria, mildly inhibits non-dermatophytic onychomycosis pathogen, and has no effect on dermatophytes (Chiang et al., 2013).

In ancient times, the tribals used roots of *S. callosus* for the treatment of inflammatory disorders and its stem bark in formulations for painful and ineffectual attempts to urinate or defecate (Jain & Defilipps, 1991). The roots of *S. cilliatus* were used for the treatment of rheumatalgia, limping, chest congestion, strangury, fever, leucoderma, skin diseases and inflammation (Warrier et al., 1994). Its stem is widely used for whooping cough, bronchitis, dropsy, leprosy and pruritus (Thomas et al., 2000). Indigo Naturalis is used from earlier times as textile dye and paint pigment. Modern research has revealed that Indigo Naturalis is used as an antipyretic, anti-inflammatory,

antiviral, antimicrobial, anticancer and detoxicant agents (Fatima et al., 2007). *Strobilanthes* is a good source of Indigo Naturalis, which is used in Chinese medicine also (Chiang et al., 2013).

S. cilliatus leaves are rich in potassium. Lupeol derived from its root is used to treat skin problems and post-delivery treatments. The plant juice is taken as an antidiabetic drug (George et. al., 2017). It is good against toothache too. The essential oil from *S. crispus* can be used as a nutraceutical towards degenerative diseases (Rahmat et al., 2006b). The high content of calcium carbonate in the plant makes the boiled water mildly alkaline and function in ease of urination (Noraida, 2005). A survey in Malaysia revealed its application to treat kidney stones by placing heated leaves on hips (Ong & Norzalina, 1999). Traditional medicine in Malaysia and Indonesia uses filtrates of boiled *Strobilanthes* leaves as antidiabetic, diuretic, antilytic and laxative (Bakar et al., 2006). Blood sugar, leprosy, urinal problems, jaundice, inflammation, and excess menstruation can be treated using *S. heyneanus* roots and leaves (Sundaram et al., 2021).

Da-Ching-Yeh is the local name for *S. cusia* in China and is used in Chinese traditional medicine. It is widely used against influenza, epidemic cerebrospinal meningitis, encephalitis B, viral pneumonia and mumps and to treat sore throat, aphthae and inflammatory diseases with redness of the skin, *etc*. The extract of the plant suppressed the writhing responses of mice and it reduced the paw edema induced by carrageenan in rats. It potently attenuated pyrexia induced by lipopolysaccharide (Ho et al., 2003).

The genus, *Strobilanthes* has been widely used in traditional herbal practices of India, China, Indonesia, *etc*. Moreover, its leaf extract can minimize the glucose level in blood and also reduces the risk of heart muscle/cardiovascular ailment (Fadzelly et al., 2006). Empirical shreds of

evidences support the use of traditional herbs that cured the disease of thousands, but the role of many of these herbs remains largely lacking.

II. Chromosome analysis

Cytotaxonomy is the prodigious son of taxonomy and cytogenetics is the science that discover the evolutionary bond between related species (Guerra, 2012). It acts as the basement for the taxonomic positions of many reviewed taxa. Recent advancements such as molecular cytogenetics, genomics and phylogenetics gave a new path to evolutionary studies. These techniques throw light on the ultrastructure of chromosomes and their mechanisms (Lysak et al., 2006). The genetic diversity of new cytotypes and morphotypes can be revealed with the help of cytomorphology (Sharma et al., 2013).

Even though many cytological works have been reported in Acanthaceae, feeble reports of South Indian species of *Strobilanthes* are available. The earlier works on karyotype analyses in the Acanthaceae suggests it as a polybasic family, which shows a continuous series of basic numbers between x = 9 and x = 40 (Govindarajan & Subramanian, 1983; 1985). They considered the basic chromosome number of *Strobilanthes* genus as 10 with *S. isophyllus* as a diploid species. But it is not true for all the species in the taxa (**Table 1**). According to Govindarajan and Subramanian (1983; 1985) *S. dyerianus*, *S. lawsoni*, *S. wightianus* and *S. pulneyensis* were euploids. Whereas *S. luridus*, *S. papillosus*, *S. barbatus*, *S. zenkerianus*, *S. homotropus* and *S. papillosus* were aneuploids.

The different populations of the ornamental plant *S. alatus* revealed two different cytotypes. They were 2n = 2x = 16 and 2n = 4x = 32. When comparing with the karyomorphological parameters, tetraploid cytotypes showed high indices with that of diploid one (Gupta et al., 2012). The

chromosome number in *S. glandulifera* and *S. tashiroi* were reported to be 2n = 30 (Kanemoto, 2001). In these species the interphase nuclei found to be of the were simple chromocenter type. Size variation in metaphase chromosomes with a pair of satellite chromosomes and mitotic prophase chromosomes of the proximal type were some of the other common karyomorphological features shared by the two species. The most common haploid chromosome number report is n = 16. The other ones were n = 9, 11, 13, 14, 15, 16 and 20. The detailed chromosome count reported so far in the genus *Strobilanthes* are recorded (**Table 1**).

Mixoploidy or polysomaty in the genus has been reported by Reshmi and Thoppil (2021) in *S. virendrakumarana*. In this species a symmetric mixoploid karyotype with three ploidy levels such as 2n = 10, 2n = 20 and 2n = 30 were observed. The normal somatic chromosome number was found to be 2n = 2x = 20 with a basic chromosome number of 10. By increasing the chromosome number, plants get a greater variability and better adaptation capability (Tena-Flores et al., 2014). Mixoploidy in plants is maintained by their capacity for vegetative propagation and apomictic propagation (Amosova et al., 2015).

In other words, the presence of more than one chromosome number in a cellular population is called mixoploidy (Pierre et al., 2011). Polyploid mixoploidy is the most common mixoploidy seen in Angiosperms (Ranjbar et al., 2011). By increasing the chromosome number by fragmentation, variability and adaptability will be acquired (Mola & Papeschi, 2006). When plants are exposed to abiotic stresses, karyotypes tend to evolve polyploidy which is suitable for the environment (Mayrose et al., 2010).

The accessory chromosomes or B-chromosomes (Bs) were first reported in a plant bug insect Metapodius by Wilson and described it as supernumerary chromosomes. In Angiosperms, more than 500 species were

reported to have Bs (Jones, 2017). It is considered as a significant component of the eukaryotic system because they are found in many at the population level (Blagojevic´ et al., 2007). Chen et al. (1993) stated no relationship between Bs with ecological and climatic conditions. Bs are derived from standard A chromosomes or foreign ones by interspecific hybridization. It has been suggested that Bs are totally or partially heterochromatic, inert but sometimes may have genes that affect recombination (Dwivedi & Kumar, 2019). The major differences between A chromosomes and Bs include the latter rarely carry active genes, it can disturb Mendelian inheritance and they neither pair nor recombine with normal complements (Houben & Carchilan, 2012). There are previous reports which reveal that Bs may interfere with various biosynthetic pathwaysthereby affecting essential oil formation (Thoppil & Jose, 1995; Mani & Thoppil, 2005). Several suggestions have been made on the presence of Bs such as they are restricted to diploids only, complete absence in tetraploids and restricted to polyploids (Dwivedi & Kumar, 2019).

Table 1: Reports of chromosome number in different species *Strobilanthes*

Sl. No.	Species	Number	Authors	Year
1.	*S. alatus*	n = 16	Bir & Saggoo	1981
		n = 16	Bhat et al.	1975
		n = 16	Vasudevan	1977
		n = 16	Mehra & Gill	1968
		n = 16	Gupta et al.	2012
		n = 32	Gupta et al.	2012
		n = 16	Marhold & Kučera	2017
2.	*S. anamallaica*	2n = 1x= 10	Reshmi & Thoppil	2021
		2n = 2x=		

		20 2n = 3x= 30		
3.	*S. anisophyllus*	n = 13	Verma & Dhillon	1967
4.	*S. asperrimus*	n = 16	Devi & Mathew	1997
5.	*S. atropurpureus*	n = 16	Saggoo	1983
		n = 16	Daniel & Chuang	1998
		n = 16	Vasudevan	1977
		n = 32	Sharma et al.	2013
6.	*S. barbatus*	2n = 32	Govindarajan & Subramanian	1985
7.	*S. callosus* Nees	n =14	Saggoo & Bir	1982
8.	*S. cilliatus*	2n = 28	Renjana	2014
9.	*S. consanguineus*	n = 16	Saggoo & Bir	1982
10.	*S. cusia*	2n = 32	Ge et al.	1987
		2n = 32	Iwatsubo et al.	1993
11.	*S. dalhousieanus*	n = 9	Verma & Dhillon	1967
		n = 9	Bir & Saggoo	1981
		n = 9	Vasudevan	1977
12.	*S. discolor*	n = 11 2n = 22	Löve	1980
		2n = 30	Grant	1955
13.	*S. dyerianus*	n =15	Daniel & Chuang	1998
14.	*S. glandulifera* Hatus.	2n = 30	Kanemoto	2001
15.	*S. glutinosus*	n = 16	Marhold & Kučera	2017
16.	*S. heteromallus*	n =16	Devi & Mathew	1997
17.	*S. heyneanus*	n = 16	Devi & Mathew	1997
18.	*S. homotropus*	2n = 42	Govindarajan & Subramanian	1983
19.	*S. isophyllus*	2n = 20	Grant	1955
20.	*S. japonica*	2n = 30	Iwatsubo et al.	1993

21.	*S. kunthianus*	2n = 32	Govindarajan & Subramanian	1983
		n =16	Devi & Mathew	1997
		n = 16	Devi & Mathew	1997
22.	*S. lawsoni*	2n = 30	Govindarajan & Subramanian	1985
		n = 20	Devi & Mathew	1997
23.	*S. lupulinus*	n = 15	Devi & Mathew	1997
24.	*S. luridus*	2n = 32	Ellis	1962
		2n = 32	Govindarajan & Subramanian	1983
25.	*S. oliganthus*	2n = 60	Iwatsubo et al.	1993
26.	*S. papillosus*	2n = 32	Raman & Kesavan	1963
		2n = 32	Fedorov	1974
27.	*S. pentastemonoides*	n = 13	Saggoo & Bir	1982
28.	*S. pulneyensis*	2n = 40	Govindarajan & Subramanian	1983
29.	*S. scaber*	n = 16	Saggoo & Bir	1983
30.	*S. tashiroi*	2n = 30	Kanemoto	2001
31.	*S. wakasana*	2n = 30	Iwatsubo et al.	1993
32.	*S. virendrakumarana*	2n = 1x = 10 + 0-1B	Reshmi & Thoppil	2021 (accepted)
		2n = 2x = 20 + 0-1B		
		2n = 3x = 30 + 0-2B		
		2n = 4x = 40 + 0-2B		
33.	*S. wallichi*	n = 16	Saggoo & Bir	1982
		n = 20	Vasudevan	1977
34.	*S. wightianus*	2n = 30	Govindarajan & Subramanian	1983
35.	*S. zenkerianus*	2n = 32	Govindarajan & Subramanian	1985

III. Phytochemical constituents

In developing countries, according to World Health Organization, 80% of the people depend upon traditional medicine for their primary healthcare and 85% of them are derived from plant extracts (Ghasemzadeh et al., 2015). The pharmaceutical activity of the herb can be correlated to the phytochemical components present in it such as flavonoids, alkaloids, phenolic acids, ester glycosides, *etc*.

S. crispus is rich in squalene, stigmasterol, sitosterol, campesterol as well as triterpenoids such as lupeol, amyrin and botulin (Cheong et al., 2016). The ethnomedical values of the species were not well known but the biological properties of many species are reported so far. The pharmaceutical quality and presence of secondary metabolites change with the environment. It was found to be decreased in North East of North Malaysia while examining the *S. crispus* leaf extract. The notable number of phenolic compounds in this plant has enhanced the anticancer effect on HeLa cells (Ghasemzadeh et al., 2015). Hence, collection locality and environmental parameters have to be considered while quantifying the phytoconstituents. Polyphenols, catechins, alkaloids, caffeine, tannins, vitamins (C, B1 and B2) and also high mineral content including potassium, calcium, sodium, iron and phosphorus were identified from this species by Maznah et al. (2000).

The Chinese medicine *Qing Dai* (Indigo Naturalis) is prepared from *S. cusia* which is used against psoriasis and for targeting interleukin-17 (IL-17). Within these formulations, three indole derivatives and seven other compounds were isolated (Lee et al., 2019). A novel isocoumarin with an unusual tetrahydro-4 H-pyran-4-one moiety fused isocoumarin core skeleton was isolated from *S. cusia* which exhibits anti-influenza virus activity *in vitro* (Gu et al., 2015). *S. flaccidifolius* which is locally known as ‘kum’ in Manipur is used as a dyeing agent. Laitonjam and Wangkheirakpam (2011) analysed

the water extracts of *S. flaccidifolius* and confirmed three indigo precursors such as indican, isatan B and isatin. In this study, three days of fermented fresh leaves gave the highest amount of indigo. In dried leaf water extract, the enzyme might have been inactivated in the drying procedure. Hence indigo was not obtained after solvent extraction with chloroform.

When female Sprague Dawley rats were administered with *S. crispus* ethanol leaf extract for two weeks, no bad effects were observed. In comparing between the treatment groups and control, no significant changes were observed regarding the serum biochemical parameters, relative organ weights, body weights, food intake and water consumption (Lim et al., 2012).

A study was conducted on five *Strobilanthes* species *viz. S. callosus* Nees, *S. ciliata* Nees, *S. integrifolia* Kuntze, *S. ixiocephala* Benth. and *S. heyneanus* Nees. From the leaf and stem extracts, a total of 43 compounds were revealed of which 13 were found to be common in leaves and stems across the species. In both the leaves and stems, lupeol was the major bioactive compound (Fernandes & Krishnan, 2019b). *Strobilanthes* species show variations in phytoconstituents in the pre and post-flowering period. The extracted oil from the post-flowering plants of *Strobilanthes callosus* contained trans-sabinene hydrate, cis-sabinene hydrate, terpinen-4-ol, α-terpineol and methyl chavicol. Whereas the oil from pre-flowering plants contain all four compounds except methyl chavicol (Weyerstahl et al., 1992).

Anticestodal drugs are used mainly against tapeworms. With the help of *Hymenolepis diminuta (*rat tapeworm) experimental model, anticestodal efficacy of *S. discolor* leaf extract was investigated. It was analysed by checking the eggs per gram of faeces counts and percentage worm recovery rates. It was followed by treating different groups of rats infected by *Hymenolepis diminuta* with methanol leaf extract. There was a significant reduction in both parameters considered. Interesting results were obtained in

the larval stages of the parasite. In this stage, not a single worm had escaped from the 800 mg/kg dose, which is given twice daily for a continuous three days. These findings suggest the high potential of the plant against cestodes and support its use in folk medicine **(**Tangpu & Yadav, 2006).

The GC-TOF mass spectroscopy of the methanolic and aqueous extracts of *S. crispus* dried leaves of the plant revealed potential phytoconstituents (Muslim et al., 2010). About 32 compounds were identified such as 3-octadedecene, α-sitosterol, campesterol, hexadecanoic acid methyl ester, lupeol, phytol and stigmasterol in the methanolic extract while in the aqueous extract, 3,5-dithiahexanol 5,5-dioxide, cyclobutanol, hydrazine carboxamide, monoethanolamine, n-propyl acetate and undecane have been identified. Moreover, the plant leaf contains, ester glycosidic compound of caffeic acid (a verbascoside), voumaric acid, caffeic acid, vanilic acid, ferulic acid and syringic acids (Cheong et al., 2016).

The wound healing capacity of *S. crispus* was studied in the rat. The wounds dressed with leaf extracts showed less inflammatory cells and high collagen in histological analysis (Al-Henhena et al., 2011). The antiviral activity of human coronavirus NL63(HCoV-NL63) using extracts of of *S. crispus* leaf and its major components revealed a positive result. Among the components, trypanthrin and indigodole B (5a R-ethyl trypanthrine) were potential against viral activity and thereby reduces the cytopathic effect and progeny virus production (Tsai et al., 2020). The HPLC analysis of *S. crispus* opens up eight The compounds from *S. crispus* will cohere the proteins that bind to the active part of the reverse transcriptase. It inhibits retrovirus proliferation which causes acquired immune deficiency syndrome (AIDS) and Adult T-cell leukemia (Endrini et al., 2007). The isolated and identified compounds in the taxa are summarized (**Table 2).**

The synergistic activity of alkaloids, steroids, terpenoids, phenols, flavonoids, tannins, saponins, glycosides and carbohydrates are together responsible for the antioxidant and antibacterial efficacy of *S. heyneanus* (Sundaram et al., 2021). A significant amount of phenols, flavonoids and tannins were observed in aqueous plant extract rather than ethanol extract (Kavitha and Indira, 2016). In *S. cilliatus*, triterpenoids were present in petroleum ether and benzene-soluble parts, flavonoids and phenols in water and alcohol-soluble parts whereas, mucilage was present in water-soluble parts only (Shirwaikar et al., 2018).

Table 2: Phytochemical compounds from different species of *Strobilanthes*

Sl. No.	Plant	Compounds identified/isolated	Reference
1.	*S. callosus*	Taraxerol, Taraxasterol, Indomethacin	Singh et al., 2002
2.	*S. cilliatus*	4-Acetyl-2, 7-dihydroxy-1, 4, 8-triphenyl-octane-3, 5-dione	Reneela & Sripathi, 2011
3.	*S. cilliatus*	Lupeol, Stigmasterol, Betulin, Stigmasterol glycoside	Reneela & Sripathi, 2010
4.	*S. crispus*	p-Hydroxy benzoic acid, p-Coumeric, Caffeic acid, Vanillic acid, Gentisic acid, Ferulic acid, Syringic acid	Soediro et al., 1987
5.	*S. crispus*	Verbascoside	Soediro et al., 1983
6.	*S. crispus*	Verbascoside	Ahmed, 1999
7.	*S. crispus*	Stigmasterol, Tetracosanoic acid, 1-Heptacosanol	Koay et al., 2013
8.	*S. crispus*	Tritriacontane, Stigmasterol	Afrizal, 2008
9.	*S. cusia*	Lupeol, Betulin, Lupenone, Indigo, Indirubin, 4(3H)-quinazolinone, 2,4 (1H, 3H)-quinazolinedione	Li et al., 1993
10.	*S. cusia*	Indole alkaloid glycoside, Indole alkaloid glucoside, Phenylethanoid glycoside	Gu et al., 2014
11.	*S. cusia*	Tryptanthrin	Honda & Tabata, 1979

12.	*S. cusia*	Isocoumarin	Gu et al., 2015
13.	*S. formosanus*	3′-Hydroxy-5,7-dimethoxyflavone 4′-*O*-β-D-apiofuranoside; 5,7-Dimethoxyflavone 4′-*O*-[β-D-apiofuranosyl(1→5)-β-D-glucopyranoside]; 4′-Hydroxy-5,7-dimethoxyflavone; 2, 6 -Dimethoxy-1,4-benzoquinone; Lupeol; Betulin	Kao et al., 2004
14.	*S. crispus*	Catechin; Epicatechin; Rutin; Myricetin; Luteolin; Apigenin; Naringenin; Kaempferol	Liza et al., 2010
15.	*S. kunthiana*	9,12-Octadecadienoic acid (Z,Z); Hexadecanoic acid methyl ester; 9-octadecenoic acid; Methyl ester; Heptadecanoic acid 16-methyl-, methyl ester; 2,6-bis(1,1-dimethylethyl)-4-methyl phenol; 3-methyl-2-ketobutyric acid tbdms, ; 2,2,3,4-Tetramethyl-5-hexen-3-ol; N-(tert-butoxycarbonyl)-2-(4-methoxyphenyl) allylamine; Cyclotrisiloxane hexamethyl; Benzenesulfonamide	Prabakaran, & Kirutheka, 2018
16.	*S. crispus*	5-Hydroxymethylfurfuralpalmitic acid; Octadec-9-enoic acid; Cyclopentadecane, Linoleic acid; Methyl 7,10,13-hexadecatrienoate; Acetic acid; Phytol; Isopropyl linoleate; Linolenoyl glycerol; Oleic acid; Elaidic acid; Glycerol oleate; Squalene	Mohamad Razak, 2019
17.	*S. crispus*	3-Octadedecyne; α-sitosterol; Campesterol; Hexadecanoic acid; Methylester; Lupeol; Phytol; Stigmasterol; 3,5-Dithiahexanol; 5,5-dioxide; Cyclobutanol; Hydrazine carboxamide; Monoethanolamine, n-Propyl acetate; Undecane	Muslim et al., 2010

IV. Pharmacognostic studies

The drugs we use nowadays are the crude form of traditional and folk medicine (Robbers et al., 1996). The medicinal quality of a plant can be assessed using pharmacognostic studies which is an important tool in plant drug designing. The adulterants in the medicinal market can be excluded with proper identification. The standardization of plant materials can be analysed using detailed microscopic evaluation. Morphological, anatomical and biochemical characteristics are the key points in the pharmacognostic standardization of the plant material. The various characteristics of a plant obtained from powder microscopy can be used as a taxonomic tool for the identification of the plant in modern taxonomy (Bijeshmon & George, 2014).

Plant research focuses on the isolation of active phytoconstituents from the extracts. These constituents can serve as marker compounds for their standardization. By resolving the biological activities, we can develop these compounds into lead molecules for further drug development. The physicochemical analysis is a worldwide accepted identification and authentication tool in pharmacognostic standardization of genuine plants.

In *S. kunthianus* the powder microscopic features revealed liberiforus fibers with thick lignified walls and they are narrow with pointed ends and lumens. The epidermal cells of the lamina possess dilated wide circular or horizontally elongated lithocyst which has a single cylindrical rod-like calcium carbonate cystolith. The frequently found cystolith in this plant has a narrow, elongated, and blunt end at one side and pointed end at the other. It was warty with 60 µm thick and 700 µm long (Balasubramaniam et al., 2020). In *S. sessilis,* the powder was pale green with a strong odor, slightly bitter, smooth and slippery in texture. The epidermis consists of diacytic stomata. The epidermal cells are polygonal and rectangular with thin, straight, walls with an abundant number of multicellular trichomes. The powder also

revealed the presence of lignified and non-lignified xylem vessels (Shende et al., 2015). The Indonesian folklore medical plant *S. crispus* is consumed by the people either fresh or boiled in water like tea. The bitter taste of the tea is due to the high content of cystoliths of calcium carbonate and the alkaline infusion. Hence it is habitually used as a medication against diabetes, kidney stones, hypertension, and constipation and also to enhance immunity to prevent the development of cancer (Ng et al., 2021).

V. BIOACTIVITIES

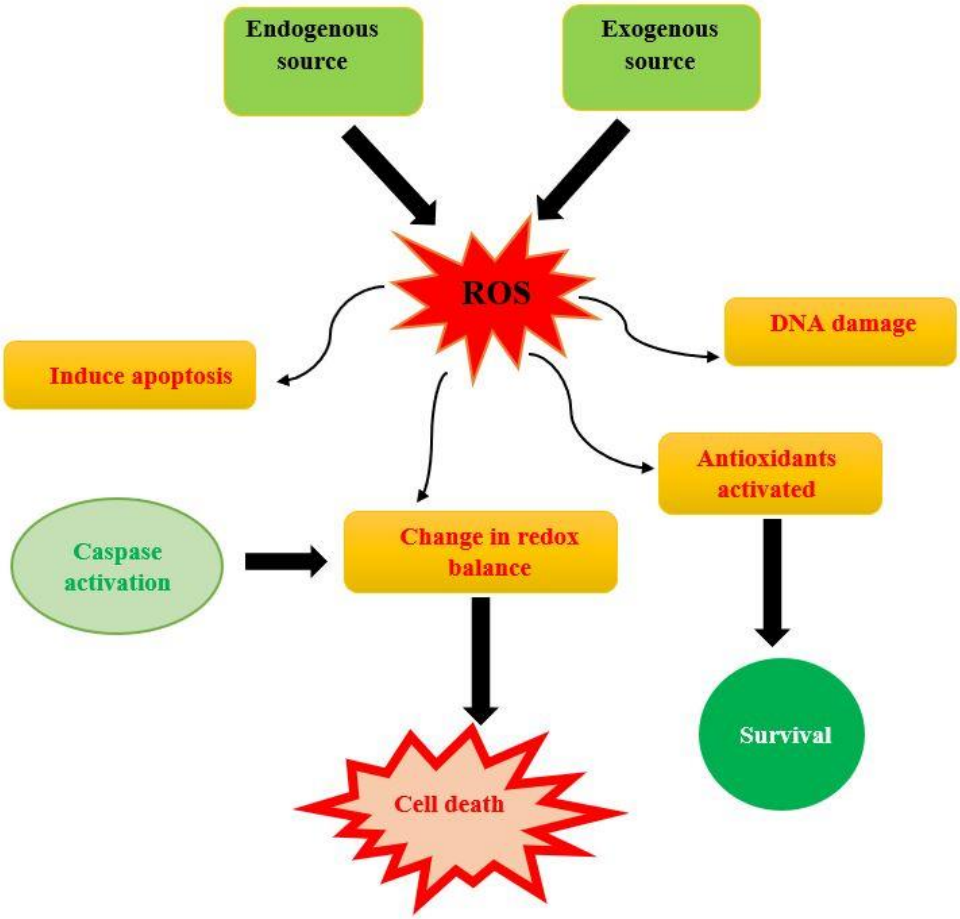
a. Antioxidant activity

Antioxidants are the only way to fight against the double-edged sword 'reactive oxygen species'. The wise use of reduction of them can delimit the diseases to an extent. Plant-derived secondary metabolites such as flavonoids, phenolic acids, and tannins are well-known scavenging activators against reactive species. The oxidative human diseases such as cancer, tissue damage and DNA injury can be controlled with the help of antioxidants. The ability of phytocompounds against these necessary evils are well researched and discussed from time immemorial. Any substance which is present in low concentrations compared to an oxidizable substrate and delays or inhibits the oxidation of that substrate is called a biological antioxidant (**Figure 1**). An ideal antioxidant at the physiological level will be completely absorbed by the body and undertake quenching of free radicals or chelate redox metals (Halliwell & Gutteridge, 1999).

Reactive oxygen species (ROS) are of both free radical and non-free radical oxygen-containing species, which increase oxidative stress and impair the redox balance (Poljsak et al., 2013). They include hydrogen peroxide (H_2O_2), superoxide (O_2-), singlet oxygen (½O_2), hydroxyl radical (·OH) and reactive nitrogen, iron, copper, and sulphur species (**Figure 2**). Among them,

hydroxyl radical is a potent ROS that causes cell damage by reacting with phospholipids of the cell membrane. Superoxide is a weak one that produces hazardous hydroxyl radicals and singlet oxygen (Alam et al., 2013). According to Sies (1991) the oxidative stress is a disturbance in the prooxidant to antioxidant balance in service of the former, leading to potential damage. The oxidative stress experienced by a cell depends on the activity of ROS generated and the ROS scavenging system. For favoring mild oxidative stress, the balance between prooxidant and antioxidant substances is kept slightly in favor of prooxidant products (Poljsak et al., 2013).

Figure 1: Mechanism of reactive oxygen species



The consumption of leaf extract of *S. crispus* as herbal tea increased the defense system especially towards degenerative diseases due to the high number of water-soluble vitamins (Maznah et al., 2000). The wide spectrum of activities of *S. tonkinensis* includes antiviral, antitumor, anti-inflammatory and anticoagulant (Chen et al., 2013). The plant has been suggested as a potential resource of squalene (Yang et al., 2014). The aqueous extracts of *S. crispus* leaves collected from North-east Malaysia (Kelantan) showed 73.8% in DPPH radical scavenging assay and 267.5 µM of Fe (II) activity in ferric reducing antioxidant power assay (FRAP) with an IC_{50} value of 44.1 µg/mL (Ghasemzadeh et al., 2015).

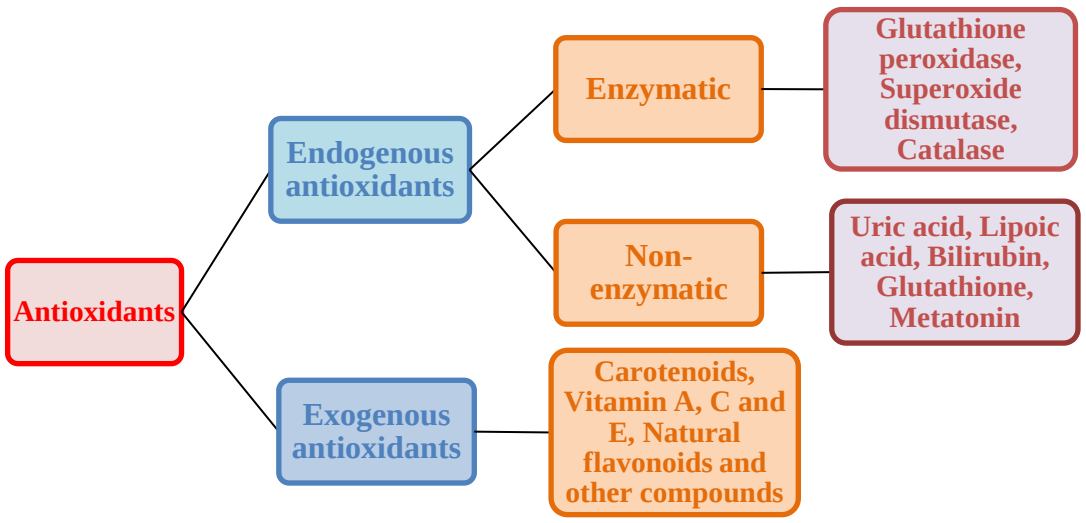
The highest percentage of inhibition in antioxidant property of *S. kunthianus* ethanolic extract (79.23±0.37) was 250 µg/mL and followed by methanolic extract (90.35±0.54) against ascorbic acid as standard (Prabakaran & Kirutheka, 2018). They suggested it as a herbal alternative for various diseases. In *S. barbatus*, the concentration of the extract for fifty percentage inhibition for DPPH, superoxide and hydroxyl radical scavenging activity were 15, 250 and 525 µg/mL respectively (Subbulekshmi et al., 2015).

The reduced awareness of pain is called analgesia. The analgesic effect of ethanolic extracts of *S. cilliatus* against the standard pain killer diclofenac is proved by the tail clip method in Swiss Albino mice (Mathew et al., 2017). A strong correlation between antioxidant activity and the total phenol contents was proposed by Qader et al. in 2011. *S. crispus* extracts showed non-toxic effects against a normal human lung fibroblast cell line (Hs888Lu). The ethanolic extracts contain high antioxidant activities compared to aqueous extracts in 1,1-diphenyl-1-picrylhydrazyl (DPPH) and FRAP assays. The methanolic extracts of *S. crispus* offer a richer source of dietary antioxidants (Tan et al., 2020). The convective dried *S. crispus* showed potential for preserving antioxidant constituents by achieving the highest antioxidant

values that were significantly higher than those obtained after freeze-drying (Chua et al., 2019).

The antioxidant properties of aqueous and methanolic extracts were studied using DPPH free radical, xanthine oxidase activity and β-carotene-linoleate model system in *S. crispus* by Muslim et al. (2010). The inhibitory activity of the *S. crispus* extracts towards the xanthine oxidase enzyme was high but they demonstrated moderate antioxidant properties. It can be witnessed by the quenching of the DPPH free radical and preventing the bleaching of β-carotene by linoleic acid. The higher antioxidant activity of methanolic extract of *S. glutinosus* can be correlated to the high content of phenol (247.85 mg GAE/g extract) and flavonoid (71.91 mg QAE/g extract). But the *n*-hexane extract exhibited the highest anti-urease activity with IC_{50} value of 0.244 mg/ml (Aziz et al., 2021).

Figure 2: General classification of antioxidants



In the case of *S. kunthiana* flowers, the ethyl acetate extract and n-butanol extract exhibited promising antioxidant activity and the n-hexane extract was devoid of any activity (Singh et al., 2014a). In the ABTS method, the ethyl acetate extracts of the root and stem of the plant showed potent *in*

vitro antioxidant activity. Among the different extracts studied, crude methanolic flower extract was the most potent one. The weak cytotoxic activity against Hep2 and HeLa cell lines was exhibited by all the extracts studied (Balasubramaniam et al., 2021). A dose-dependent activity was shown by ethanolic extract of *S. asperrimus* in serum biochemical parameters like hepatic antioxidant enzymes like glutathione peroxidase (GPX), superoxide dismutase (SOD), catalase (CAT) and malondialdehyde (MDA). When compared with the standard silymarin, significant antioxidant activity was detected by decreasing MDA and increasing SOD, GPX and CAT (Samal, 2013). The significant increase in glutathione peroxidase and superoxide dismutase activities, prove the antioxidant effect in streptozotocin-induced diabetic rats treated with *S. crispus* juice (Norfarizan-Hanoon et al., 2009).

b. Cytotoxicity in *Allium cepa*

To investigate the physical and chemical mutagenesis, *Allium cepa* test is widely used. The cytotoxicity, especially the cytogenetic and chromosome aberration can be tested using the standard protocol of *A. cepa* assay. It was first investigated by Levan in 1938 and later it was standardised by Fiskesjö and later on by Rank (Bonciu et al., 2018). *A. cepa* is an efficient standard organism for this purpose (Ramya Sree & Thoppil, 2018). Due to their sensitivity and good correlation with mammalian test systems, it is routinely used to check the genotoxic potential. The assay was used for evaluating the genotoxic potential of environmental and industrial effluent samples.

Mitotic index is an acceptable measure of cytotoxicity (Smaka-Kincl et al., 1996). The lowering of the quantitative estimation of the mitotic index suggests the suppression of mitotic activity in *A. cepa.* It may be due to the cell cycle blockage at G2 phase or DNA synthesis inhibition (Prajitha & Thoppil, 2016). The mitodepressive efficiency of the extract can be evaluated by this method (Reshmi & Thoppil, 2020). Along with this, the abnormality

percentage increases accordingly. The extract/chemical induces several chromosomal aberrations in the cell which is an indication of the toxicity of the same.

In the genus *Strobilanthes*, few reports on *A. cepa* assay were available in the literature. But the assay has been widely used and accepted by researchers due to the worthwhile results in other plants. With its sensitivity, wide availability and geographical distribution, *A. cepa* can be selected as a suitable candidate for exposure analysis.

A dose-dependent cytotoxicity was observed in methanolic extract of *S. virendrakumarana*. The major clastogenic aberrations detected were nuclear lesions, pulverized ball metaphase, chained metaphase, coagulated anaphase, nuclear budding, etc. Sticky stathmo-anaphase, pole to pole metaphase, scattered metaphase, tropokinesis at metaphase, vagrants at anaphase and cytostasis were some of the non-clastogenic aberrations obtained. A positive correlation of concentration of the extract and abnormality percentage was detected (Reshmi & Thoppil, 2019). In *S. heyneanus* the aberrations resulted were chromosome lesions, anaphase bridges, C-mitosis, pulverization, stathmo-anaphases, diagonal orientation, chromosome fragments *etc*. With the with increasing concentrations of the extract and longer durations of exposure the total percentage of aberrations as well increased (Renjana & Thoppil, 2013).

c. Anti-proliferative activity

The use of phytocompounds to protect uncontrolled cell growth has immense importance in the modern world due to the alarming increase of various dreadful diseases. Plants and plant-derived compounds have been investigated for the past several years to counteract against the diseases as a natural alternative.

The oil extracted from the aromatic herb, *S. heyneanus* is effective in inflammation treatments (Nayar et al., 1988). Agarwal and Rangari (2003) showed anti-inflammation activity of lupeol and α-lupeol isolated from *S. callosus* and *S. ixiocephalus* respectively. Li et al. (1993) showed the anticancer activity of the indole alkaloid, indirubin isolated from *S. cusia*. Even though *S. sessilis* is not suitable for human consumption due to its toxicity, it is used by the local Adivasi tribals and villagers for inflammatory diseases (Shende et al., 2015). From the aerial parts of *S. cusia,* three indole alkaloid glycosides, strobilanthosides A-C, two known indole alkaloid glucosides and five phenylethanoid glycosides were isolated (Gu et al., 2014).

The high anticancer activity of *S. crispus* is due to its phytochemical constituents especially mineral contents, antioxidant vitamins as well as catechin (Maznah et al., 2000). The findings of Chong et al. (2012) suggest that its leaf extract can induce apoptosis and DNA fragmentation on hormone-dependent breast cancer cell lines. Moreover, it is powerful in reducing hepatic necrosis in rats by inhibiting the enzymes involved in boosting carcinogens (Hanachi et al., 2008). But its crude leaf extract, as well as essential oil, were reported to be non-toxic to normal Chang liver cell line (Rahmat et al., 2006a; Rahmat et al., 2006b). The anti-proliferation of water extracts of *S. crispus* towards retroviruses is due to the presence of compounds with high binding affinity to protein molecules (Kusumoto et al., 1992). Among the subfraction of the dichloromethane extract of *S. crispus,* SC/D-F9 constantly killed breast and prostate cancer cell lines but it doesn't affect the normal breast cells MCF-10A. This fraction exhibited higher cytotoxicity when compared with tamoxifen, paclitaxel, docetaxel and doxorubicin. It induced apoptotic cell death with the help of caspase 3 and/or 7. Hence the plant has potent anticancer activity and can be studied further (Yaccob et al., 2010).

Endophytic fungi have been used beneficially in various fields such as pharmaceutical, agricultural and biotechnological applications due to the presence of secondary metabolites (Strobel & Daisy, 2003). Jinfeng et al. (2017) have isolated two endophytic fungi from *S. crispus* using potato dextrose agar medium which shows antimicrobial and anticancer activities. They were named as (PDA) BL3 and (PDA) BL5. The anticancer studies were performed in human prostatic adenocarcinoma cells, human hepatocellular carcinoma cells, human alveolar adenocarcinoma cells, human colorectal adenocarcinoma cells, and human breast adenocarcinoma cells. Among the two isolates (PDA) BL5 has high anticancer activity when compared with (PDA) BL3.

The potential death of MCF-7 cells was characterized by cell regulation, DNA degradation, cytochrome c release and caspase activation. The ethanolic extracts of *S. crispus* were apoptotic for p53, cdk2 protein and caspase 3/7 and downregulation of XIAP protein which is an apoptotic inhibitor (Chong et al., 2012). The antiglycolytic activities of *S. crispus* fraction and its bioactive components on triple-negative breast cancer cells (MDA-MB-231) are attributed to the bioactivity of the plant (Muhammad et al., 2021). The plant was cytotoxic against human liver cancer (Hep G2) and breast cancer (MCF-7) with an IC_{50} value of 0.3 and 24.8 µg/mL. But it was not toxic against colon cancer cell Caco-2. The C-myc is a proto-oncogene that is an indicator of cell proliferation and it is expressed in cancer cell lines. So, its downregulation is mandatory for inducing apoptosis. The crude extract of *S. crispus* suppressed the C-myc gene (Endrini et al., 2007). The studies in oral toxicity effect of *S. crispus* in ethanolic leaf extract was done in 20 female Sprague–Dawley rats. In the two weeks experiment no signs of toxicity, lethality, and abnormal behavioural changes were observed (Lim et al., 2012). The phytocompound γ-sitosterol isolated from *S. crispus* was tested on Caco2 cells. It exerted strong anti-proliferative ability with an IC_{50} value of 8.3 µg/mL (Endrini et al., 2014).

The blue hill slopes of Western Ghats are due to the blooming by *S. kunthianus* once in twelve years. It is a shrub that grow abundantly in the Shola Forest of the Western Ghats in South India (Paulsamy et al., 2007). The ethanolic extract of *S. kunthianus* and *S. cuspidatus* showed *in vitro* anti-inflammatory and anti-osteoarthritic activity. *S. kunthianus* was tested in the RBC membrane stabilisation method whereas *S. cuspidatus* was done in the Rabbit cartilage explants culture method (Desu et al., 2011a). In another study (Desu et al., 2011b), the same species was analysed for its anti-inflammation in 'carrageenan induced rat edema method' and 'cotton pellet induced granuloma formation' in rats.

The *in vitro* analysis of ethyl acetate and n-Butanol flower extracts of *S. kunthianus* proves that it is a promising free radical scavenger in DPPH and H_2O_2 radical scavenging assays (Singh et al., 2014a). In *in vivo,* the histopathological evaluation of kidney and liver tissues of rats revealed the antioxidant and hepatoprotective effect of methanolic extract of *S. kunthianus.* The unique components in the plant are having anti-inflammatory, antigiardial, anti-osteoarthritic, analgesic, antioxidant, antibiofilm, enzyme inhibitor, central nervous system depressant, antifungal, antibacterial, antiseptic, antimicrobial, anticancer, cytotoxic, hypocholesterolemic 5-alpha reductase inhibitor activities and protect skin against UV (Paulsamy et al., 2007; Balasubramaniam et al., 2020).

S. callosus has protective efficacy towards acute and chronic inflammation in rat models (Kumar et al., 2013). They administered ethanol, chloroform and petroleum ether extracts in different doses (100, 200 and 400 mg/kg). The inflammation was induced by carrageenan and Freund's complete adjuvant model in the plantar surface of rats. In the carrageenan-induced model, petroleum ether extract showed a significant effect whereas in Freund's complete adjuvant model, both extracts were found to be effective. *In vivo* protection of CRC of *S. crispus* extract was done in HT29 cell lines (Al-Henhena et al., 2015b).

According to Fernández et al. (2001) pentacyclic terpenes could decrease edema formation. Roots of *S. callosus* and *S. ixiocephala* have long been used for inflammation disorders in folk medicine (Agarwal & Rangari 2003) and their medicinal property is thought to be conferred by pentacyclic terpene, lupeol. Baraya et al. (2019) studied the anti-migration, anti-invasion and anti-metastasis effects of *S. crispus* leaves on breast cancer cells (MDA-MB-231) by using a sub-fraction F3. The fraction contains β-sitosterol, stigmasterol, campesterol, lutein, pheophytin a, 131-hydroxy-132-oxo-pheophytin a, and 132-hydroxy-pheophytin a. Thus, the fraction exerted an anti-proliferative effect with the IC_{50} value of 84.27 µg/mL after 24 h of exposure and 74.41 µg/mL after 48 h of exposure. Similarly, the subfraction F3 from *S. crispus* was capable of triggering the immune system in rats-bearing NMU-induced mammary tumor. So, this may support the traditional use of leaves of the plant to boost the immune system (Yankuzo et al., 2018). Another study conducted by Gordani et al. (2017) investigated the anti-proliferative effects of *S. crispus* on MCF-7 cells using different extracts of leaves and the stem (methanol, hexane, chloroform, ethyl acetate, and aqueous extracts). The essential oil from *S. crispus* did not show any effect on both MCF-7 and MDA-MB-231 cells (Rahmat et al., 2006b).

The dichloromethane fraction (F3) of *S. crispus* was evaluated for the immunomodulatory effects. The administration of F3 enhanced the expression of CIITA and MHC-II on the mammary cancer cells and the number of infiltrating CD4+ and CD8+ immune cells. Along with that after 2 months, the serum level of chemokine ligand 2 (CCL2) decreased significantly while the level of interferon-gamma (IFN-γ) increased (Yankuzo et al., 2018). Among the five different leaf extracts of *S. crispus* studied (hexane, chloroform, ethyl acetate, methanol, and aqueous) in CNE-1 cells, the ethyl acetate extract showed the strongest anti-proliferative effect on the cells with an IC_{50} value of 119.00 ± 48.10 µg/mL. It was followed by an IC_{50} value of 119.00 ± 48.10 µg/mL (Koh et al., 2015). The previous cell line studies conducted in the taxa are tabulated (**Table 3)**.

There are two types of lung cancers: non-small cell lung cancer and small cell lung cancer. The symptoms include a slight cough or shortness of breath and may become severe. The methanolic leaf extract of *S. crispus* was analysed for its cytotoxic effect on the NCI-H23 lung cell line. But there was no considerable effect in it with an IC_{50} value greater than 200 µg/mL (Ng et al., 2021). The methanolic extract of *S. crispus* exhibited a cytotoxic response towards the T-47D and MCF7 cells and the aqueous extract was found to be non-toxic towards all cell lines used. A notable anti-angiogenic activity was shown by both aqueous and methanolic extracts (Muslim et al., 2010). There was as significant decrease in serum glucose levels in both male and female diabetic and normal rats when treated with *S. crispus* juice. It has reduced the cholesterol, triglyceride and LDL-cholesterol level but the HDL-cholesterol decreased insignificantly in treated diabetic and normal rats. Hence the plant can be an alternative for lowering glucose, cholesterol and triglyceride for diabetic patients (Norfarizan-Hanoon et al., 2009).

Table 3: Previous reports on the cell line studies in *Strobilanthes*

Sl. No.	Plant	Cell line	Reference
1.	*S. crispus*	MCF-7	Chong et al., 2012
			Gordani et al., 2017
2.	*S. crispus*	MCF-7, MDA-MB-231, PC-3, DU-145	Yaacob et al., 2010
3.	*S. crispus*	MCF-7, MDA-MB-231	Yaacob et al., 2014
4.	*S. crispus*	CCD-841, HT-29	Al-Henhena et al., 2015b
5.	*S. crispus*	MCF-7, MDA-MB-231	Bakar et al., 2006
6.	*S. crispus*	HeLa	Chong et al., 2014
7.	*S. crispus*	MCF-7, MDA-MB-231, PC-3, DU-145	Yaccob et al., 2010
8.	*S. crispus*	MCF-7, MDA-MB-231	Yaccob et al., 2016
9.	*S. crispus*	HepG-2, MDA-MB-231	Koh et al., 2017
10.	*S. crispus*	CNE-1 NPC	Koh et al., 2015
11.	*S. crispus*	Caco-2, MDA-MB-231, HepG-2	Rahmat et al., 2006a

d. Colorectal cancer

Resistance to necrosis or apoptosis, persistent signals of cell proliferation, escape from growth-suppressing factors, uncontrolled cellular replication, sustained blood supply, and lastly capable of metastasizing are the six hallmarks of cancer (Hanahan, 2011). Phytotherapy or herbal medicine or herbalism is the use of plants extracts for either treatment or health-promoting purposes.

Colorectal cancer (CRC) occupies the third position among the reported cancers in the World. Industrialization and urbanization lead to an increase in the number of cancers. The greatest report of colon cancer is in Asia and Eastern European countries (Labianca et al., 2010). The inheritance of this cancer is very low. Apart from hereditary environmental factors, food-born mutagens, intestinal commensals and pathogens, chronic inflammation also led to metastasis (Terzić et al., 2010). According to World Cancer Research Fund and the American Institute for Cancer Research (2007), colon cancer can be prevented by proper diet and related factors. The modern habits of man including fast food and lack of physical exercise cause CRC. Calcium, milk, garlic, dietary food can protect man from cancer. According to World Health Organization (WHO), CRC is the second most damaging disease which causes approximately one million deaths in 2020 out of the ten million deaths due to cancer (https). More than a million new patients with CRC are reported in the World each year (Ahmed, 2020).

The diagnostic strategies of colon cancer include instrumental and pathological analysis, radiological techniques and biological markers. A colonoscope or sigmoidoscope can be used to perform endoscopies. It relies on vision on inflated bowel. Specimens for biopsy are taken from lesions for a primary diagnosis. The histology of the lesion can be visualized by colonoscopy. The virtual colonoscopy or Computed Tomographic (CT)

colonoscopy can be used. The early detection and diagnosis of cancer can be done using serological markers. Carcinoembryonic antigen (CEA) is a biological marker which is useful in pre and postoperative stages but with low predictive value of diseases (Fletcher, 1986).

A sequence of mutation followed by expansion is considered as carcinogenesis (Vineis, 2003). The normal colon cells divide in an asymmetric manner and add cells to the crypt's proliferative area. These cells later differentiate into Goblet cells or move towards the lumen. The genomic alterations in these cells make them divide symmetrically. Inadequate apoptosis will result in the accumulation of altered cells resulting in colonial expansion (Leubeck & Moolgavkar, 2002). Adenomatous crypts or microadenoma or dysplastic crypt foci are the precursors of the adenomatous polyps (Roncucci et al., 2000). They are in turn the precursors of colon carcinoma. The differentiation of colon cells is mediated by a microfloral environment and diet (Bry et al., 1996). The high intake of refined grain, red meat and inadequate intake of folate and methionine may increase the chance of colon cancer (Giovannucci et al., 1995). Dietary food habits can lower the risk by acting as protectors. They maintain a balance between cell division, maturation and death.

The cell cycle keeps a balance between growth-promoting oncogenes and growth-inhibiting tumor suppressor genes. Colon neoplasia is affected by two genes such as gatekeeper genes and caretaker genes. The former will regulate cell birth and death whereas the latter will direct the rate of gene mutations (Rupnarain et al., 2004). Advanced carcinoma is lethal but early detected ones are uniformly curable. The environment, as well as hereditary factors are responsible for colorectal cancer (CRC). The early detection and effective interventions for the late stage of this public health burden have no improvement. The number of colonic aberrant crypt foci has decreased in

azoxymethane-induced CRC when *S. crispus* was orally administered. It has down-regulated the expression of PCNA, Bcl-2 and β-catenin. Moreover, it inhibits the malondialdehyde and nitric oxide levels whereas stimulates catalase and guaiacol peroxidase activities. By suppressing the early and intermediate carcinogenic phases, *S. crispus* proves its ability against CRC (Al-Henhena et al., 2015b).

A combination of chemotherapies like 5-fluorouracil (5-FU), oxaliplatin or irinotecan with some positive effects can be increased by combination with vascular endothelial growth factor receptor (VEGFR) or epidermal growth factor receptor (EGFR) treatments (Saltz et al., 2008; Douillard et al., 2014). Tumour heterogeneity can be analysed with the recent developments in single-cell techniques. There is a similarity between human lung cancer and CRC with that mouse tumours. With the help of single-cell technologies, a better cross-comparison of human and mouse tumours can be achieved (Lannagan et al., 2021).

Depending on the location of the tumor, CRC shows differences in incidence, pathogenesis, molecular pathways and outcome. The genetic and epigenetic changes due to inherited and environmental factors make differences in right-sided colon cancer and left-sided colorectal cancer. Hence, special care must be given to patients to determine the individual variations in biological and molecular characteristics of CRC (Lee et al., 2015).

MATERIALS AND METHODS

I. PLANT MATERIALS

The three species used for the study were *Strobilanthes anamallaica* J. R. I. Wood, *Strobilanthes hamiltoniana* (Steud.) Bosser & Heine and *Strobilanthes virendrakumarana* Venu & P. Daniel (**Plate 1**). Among them, *S. anamallaica* and *S. virendrakumarana* are indigenous species of South Western Ghats, but they are easily available in their endemicity. For a comparative study of these aboriginal species, *S. hamiltoniana* which is a proven medicinal plant was also included. Except for *S. hamiltoniana,* which flowers throughout the year, the other two species have plietesial nature of flowering. All the specimens were collected from different parts of Kerala. *S. anamallaica* was collected from the Nelliyampathy hills of Palakkad district. Whereas *S. hamiltoniana* was procured from Wayanad and *S. virendrakumarana* was from Bhothathankettu of Ernakulam district. The specimens were taxonomically identified and authenticated with the help of Dr A. K. Pradeep, Assistant Professor, Department of Botany, University of Calicut and Dr K. M. Prabhukumar, Plant Diversity, Systematics and Herbarium, CSIR-NBRI, Lucknow. The voucher specimens were herbarised and deposited in Calicut University Herbarium (CALI).

★ ***Strobilanthes anamallaica*** **J. R. I. Wood (CALI: 123781)**

Flowering and fruiting: September - December

Flowering periodicity: Annual / 8 years

Distribution: Karnataka, Kerala, Tamil Nadu

Description of the plant:

It is a shrub with 1 - 2 m height having slender branches. The plant is endemic to Southern Western Ghats and common along the margins of evergreen forests at 1500 - 2000 m asl. The leaves are opposite, narrow, elliptic or lanceolate. The downward-facing white flowers represent a specific mode of pollination. The inflorescence is a spike with 2 small leaves in the middle of the peduncle. The bracts are obovate-orbicular. The calyx is 1.2 cm long with an acute/obtuse apex. The corolla is tubular-campanulate, glabrous outside and hairy inside. It has 4 stamens in didynamous condition with extended staminal hairy sheath. The ovary is placed on a prominent disc. The capsules are broad and elliptic. The seeds are orbicular, complanate and glabrous.

★ ***Strobilanthes hamiltoniana*** **(Steud.) Bosser & Heine (CALI: 123782)**

Flowering and fruiting: October - February

Flowering periodicity: Annual

Distribution: Assam, Meghalaya, Sikkim, Kerala, Maharashtra, West Bengal, Sri Lanka

Description of the plant:

It is a small shrub with few branches and shining leaves. It is native to North East India and is grown in South India as a garden plant. The colour of the corolla is unique in South Indian species. The peculiarity of the species is its annual flowering and perennial nature with continuous seasonal flowering. The quadrangular stem reaches above 1 m in height. The leaves are subequal, elliptic or ovate. The inflorescence is a panicle. The capitate flowers without bracts are sometimes scattered at the end of branches. It has 5 lobed calyx and tubular corolla with pale purple or light violet colour. The stamens are 4,

didynamous, connate at base. The ovary is found on a disc with a slightly exserted style. Oblong and glabrous capsule. The 4 seeds are ovate, complanate, pubescent, concave with minute areole.

★ ***Strobilanthes virendrakumarana*** **Venu & P. Daniel (CALI: 123780)**

Flowering and fruiting: October - March

Flowering periodicity: 10 years

Distribution: Kerala

Description of the plant:

It is a large shrub member in the genus *Strobilanthes*. It is very common in semi-evergreen and moist deciduous forests at low elevations but endemic to Kerala. It reaches up to 2 - 3 m with terete stem and faintly swollen above nodes. It has unequal leaves in each node with crenulate or serrulate at the margin and acuminate at the apex. The flowers are seen in small heads usually enclosed in 2 unequal involucral bracts. The calyx is ¼ united and lobed above. The corolla is campanulate. The stamens are 4, didynamous with prominent hairs. The ovary is minutely glandular. Capsules few in each spike, seeds are four which are orbicular, smooth with a depression on one side. The vernacular name in Malayalam is *chorukurinji* as the shape and colour of the flower bud is similar to that of cooked rice. Morphologically it can be identified easily by the yellow-red glands on the lower surface of leaves.

Plate 1: Habit of *Strobilathes* species a. *S. anamallaica*; b. *S. hamiltoniana*; c. *S. virendrakumarana*

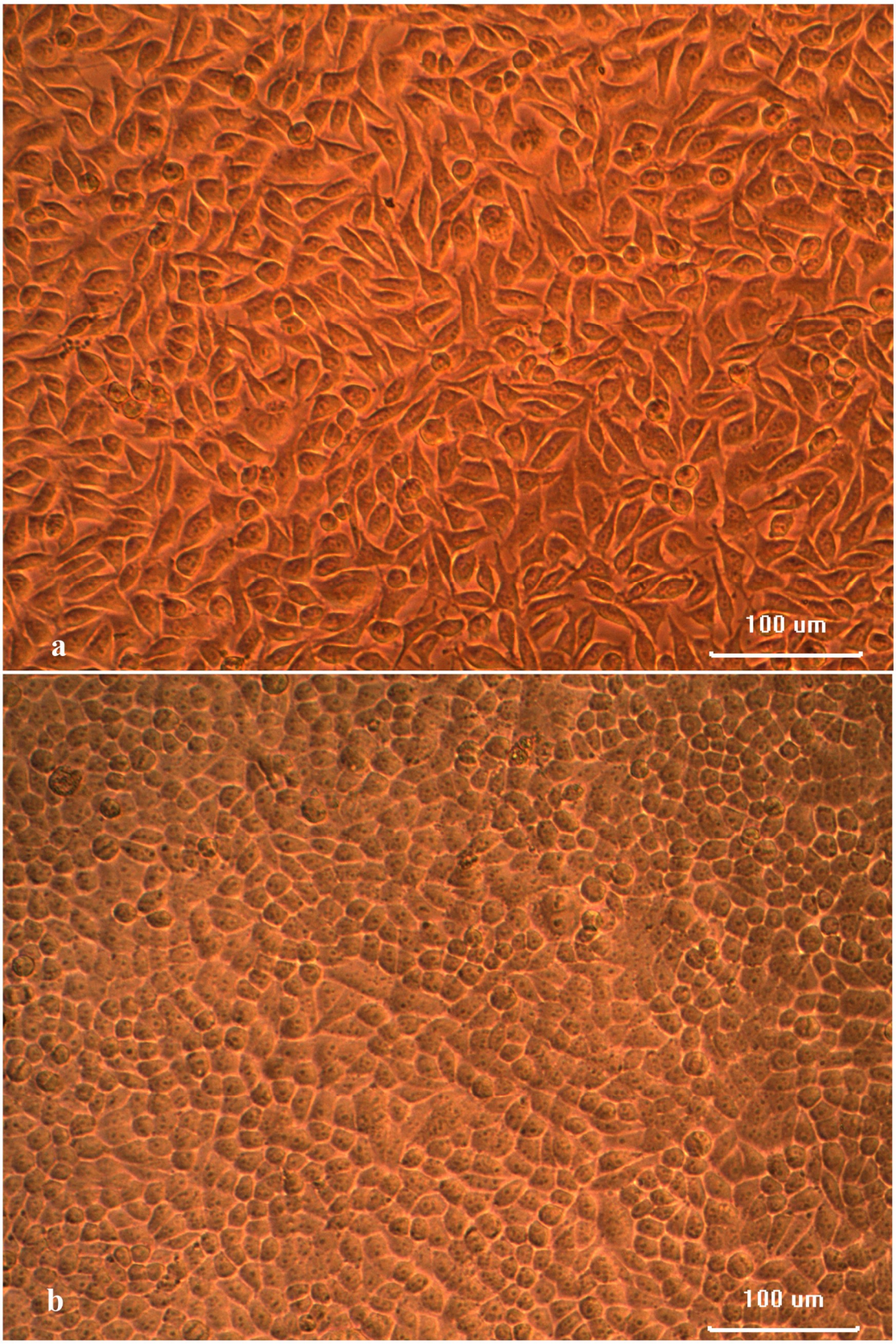


Plate 2: Cell lines under study a. L929 (Normal cell line); b. DLD1 (Human colorectal adenocarcinoma cell line)

II. METHODOLOGY

PHASE I - CYTOGENETICAL STUDIES

The study focuses on the ploidy level of the plant specimen along with chromosome number and other karyomorphometric parameters. It includes chromosome arm length, disparity index, forma percentage *etc*. The karyomorphological studies can predict the evolutionary relationship between species.

a. Mitotic squash preparation

The adventitious roots from healthy twigs were collected from fields on sunny days at peak mitotic activity time. Cleaned roots were subjected to pretreatment in chilled paradichlorobenzene at 4 °C for 5 min and thereafter for 3 h at 16-18 °C for better cytostatic effects. The pretreated roots were washed in distilled water twice and fixed in modified Carnoy's fluid (**Appendix 1**) overnight. Hydrolysis was done in 1N HCl for 15 min and later washed in distilled water. It is followed by aceto-orcein (**Appendix 2**) staining for 3-4 h. Destaining was done with 45% acetic acid. It is followed by slide preparation using squash techniques (Sharma & Sharma, 1990) in 45% acetic acid itself. Slides were observed under 100X of a light microscope (Olympus CX21FSI, binocular research microscope, Japan) and images were taken using an attached AmScope MU Series digital camera.

b. Karyomorphological analysis

Photographs of karyotypes with well-spread chromosomes having clear visibility were analysed in triplicate to prepare the karyograms and idiograms. Computer-based programs such as AutoCAD, Adobe Photoshop, Microsoft Paint and a data-based analysing system such as Microsoft Excel were used for this. False numbering was given to chromosomes and inserted into

AutoCAD. Based on the centromeric positions, the length of arms were measured and chromosomes were identified. The numbering is done in the decreasing order of chromosome lengths. On the basis of chromosome length, arm ratio and centromeric indices, homologous chromosome pairs were identified and were classified according to Abraham and Prasad (1983). With the help of the Adobe Photoshop program, paired chromosomes were subjected to karyogram and ideogram preparation.

Table 4: Chromosome nomenclature in relation to the position of centromere based on arm ratios and centromeric indices (Abraham and Prasad, 1983)

Nomenclature	Notation	R_1 (s/l)	R_2 (l/s)	I_1 (s/c%)	I_2 (l/c%)
Median	M	1.00	1.00	50.00	50.00
Nearly median	nm	0.99 - 0.61	1.01 - 1.63	49.99 - 38.01	50.01 - 61.99
Nearly submedian	nsm (-)	0.60 - 0.34	1.64 - 2.99	38.00 - 25.01	62.00 - 74.99
Submedian	SM	0.33	3.00	25.00	75.00
Nearly submedian	nsm (+)	0.32 - 0.23	3.01 - 4.26	24.99 - 18.20	75.01 - 81.80
Nearly subterminal	nst (-)	0.22 - 0.15	4.27 - 6.99	18.19 - 12.51	81.81 - 87.49
Subterminal	ST	0.14	7.00	12.50	87.50
Nearly subterminal	nst (+)	0.13 - 0.07	7.01 - 14.38	12.49 - 5.01	87.51 - 94.99
Nearly terminal	nt	0.06 - 0.01	14.39 - 19.99	5.00 - 0.01	95.00 - 99.99
Terminal	T	0.00	∞	0.00	100.00

The number of chromosomes, position of centromere as well as presence or absence of secondary constriction, *etc.* determine the karyotype of the plant. They were drawn and arranged based on chromosome size in descending order. Various karyomorphometric and asymmetry indices calculated are mentioned below:

According to Lavania and Srivastava (1992), dispersion index (DI) was determined as

$$\text{DI}\ (\%) = \frac{\text{Centromeric gradient} \times \text{Coefficient of variation}}{100}$$

Where,

$$\text{Centromeric gradient} = \frac{\text{Length of median short arm}}{\text{Length of median chromosme}} \times 100$$

$$\text{Coefficient of variation} = \frac{\text{Standard deviation of chromosome length}}{\text{Mean of chromosome length}} \times 100$$

The mean centromeric index otherwise called the total forma percentage (TF%) was calculated (Huziwara, 1962) by the formula,

$$\text{TF}(\%) = \frac{\text{Total sum of short arm length}}{\text{Total sum of chromosome length}} \times 100$$

The karyotype asymmetry index (As K%) was calculated using the following formula developed by Arano (1963).

$$\text{As K}\% = \frac{\text{Total sum of long arm length}}{\text{Total sum of chromosome length}} \times 100$$

The index of karyotype symmetry and the index of chromosomal size resemblance was developed by Greilhuber and Speta (1976) to evaluate karyotype asymmetry. These two indices were later called by Venora et al. (2002) as the Syi index and the Rec index, respectively (Paszko, 2006).

The formulae are as follows:

$$\text{Syi} = \frac{\text{Mean length of short arms}}{\text{Mean length of long arms}} \times 100$$

$$\text{Rec} = \frac{\sum_{i=1}^{n} \frac{\text{CLi}}{\text{LC}}}{\text{n}} \times 100$$

here CLi represents the length of each chromosome, LC is the longest chromosome and n is the number of chromosomes analysed.

The two numerical parameters (Romero-Zarco, 1986) to estimate karyotype asymmetry were the intrachromosomal asymmetry index (A1) and interchromosomal asymmetry index (A2). Karyotype asymmetry for the relation between the chromosome arms can be estimated using A1 whereas karyotype asymmetry due to relations between sizes of different chromosomes can be estimated using A2. A1 and A2 were calculated by the following equations:

$$\text{A1} = 1 - \left(\frac{\sum_{i=1}^{n} \frac{\text{bi}}{\text{Bi}}}{\text{n}} \right)$$

where bi and Bi represents the average length for short arms in every homologous chromosome pair or group and average length for long arms in every homologous chromosome pair or group respectively. And n is the number of homologous chromosome pairs or groups.

$$\text{A2} = \frac{\text{Standard deviation of chromosome length}}{\text{Mean of chromosome length}}$$

Watanabe et al. (1999) developed the degree of asymmetry of karyotypes (A) which is calculated using the formula,

$$\text{A} = \frac{\sum_{i=1}^{n} \frac{\text{pi} - \text{qi}}{\text{pi} + \text{qi}}}{\text{n}}$$

Here p and q are the lengths of the long arm and short arm of the chromosome respectively and n is the haploid chromosome number of the species.

PHASE II – PHYTO-PHARMACOGNOSTIC PROFILING

Phytochemical characterisation

The collected shoot system of the plants was shade dried and made into small pieces for easy grinding. The preliminary phytochemical analysis was done in three different extract of each plant prepared using distilled water, methanol and hexane. For this 5 g powdered sample were dissolved in 50 mL of each solvent. They are kept overnight for complete dissolution. These solutions were filtered using Whatman filter paper No.1 and the obtained filtrates were used for various phytochemical tests.

a. Qualitative phytochemical screening

The presence of various secondary phytoconstituents in the three different extracts were analysed using standard chemical tests (Sofowora, 1993; Trease & Evans, 2002; Harborne, 1998; Edeoga et al., 2005; Shanmugam et al., 2019). The preliminary qualitative analyses was done through various tests.

i. Tests for alkaloids

- **Wagner's Test**

A few drops of Wagner's reagent (**Appendix 3**) are added to the test tube containing 1 mL of the extract. The formation of a reddish-brown precipitate indicates the presence of alkaloids.

- **Hager's Test**

To 1 mL of the extract, Hager's reagent (Saturated picric acid solution) is added. The formation of a yellow-coloured precipitate indicates a positive test.

ii. Tests for flavonoids

- **Alkaline reagent Test**

Extracts (1 mL) were treated with a few drops of NaOH solution. The formation of an intense yellow colour that becomes colourless on the addition of a few drops of dilute acid indicates the presence of flavonoids.

- **Lead acetate Test**

A few drops of lead acetate solution were poured into 1 mL of the extract. The formation of a yellow-coloured precipitate indicates the presence of flavonoids.

iii. Test for phenolic compounds

- **$FeCl_3$ Test**

Few drops of 5% (w/v) $FeCl_3$ is added to 2 mL of the extract. It is observed for the presence of brownish-green or blue-black colour.

iv. Tests for tannins

- **Breymer's Test**

To 2 mL of the extract, 10% alcoholic $FeCl_3$ was added. Blue or greenish colour reveals the presence of tannins.

- **Ferric chloride test**

About 2 mL of the filtrate is taken and a few drops of 1% ferric chloride solution were added. The presence of tannin is shown by blue-black, green or blue-green precipitate.

v. Test for terpenoids

- **Salkowski's Test**

- About 2 mL of extracts were treated with 2 mL chloroform and filtered. Filtrates were treated with a few drops of concentrated H_2SO_4, shaken and allowed to stand. The appearance of a golden yellow colour at the interface indicates the presence of a steroid ring.

vi. Test for saponins

- **Foam Test**

2 mL of the extract was shaken well with 2 mL of water. The test is based on the production of persistent foam, indicating a positive test for saponins.

vii. Test for glycosides

- **Keller Killiani Test**

5 mL of the extract was treated with 2 mL of glacial acetic acid and a drop of 5% $FeCl_3$ was added to it. It was underlaid by a few drops of concentrated sulphuric acid. Glacial acetic acid containing 1% (w/v) $FeCl_3$ gives a brown ring in the presence of 2-deoxysugar in the glycone portion of the phytochemical.

viii. Test for phlobatannins

- **HCl Test**

About 2 mL of the extract was added to 2 mL of HCl (1%) and the mixture was boiled. A red precipitate was deposited. It is taken as a piece of evidence for the presence of phlobatannins.

ix. Test for coumarins

1 mL of each extract was treated with an alcoholic NaOH solution. The production of dark yellow colour indicates the presence of coumarins.

x. Test for resins

The extract (5 mL) was diluted with 10 mL distilled water and shaken for 4 min. The presence of resin is indicated by turbidity formation.

b. Quantitative estimation of phytochemicals

★ **Extraction**

The prerliminary assays revealed the presence of more components in methanol extract. Hence, the soxhlet extraction and further analysis were done using the solvent methanol. For this 10 g of the powder was extracted against 100 mL of methanol using the soxhlet apparatus. It requires more than 8 extraction cycles up to 6 - 8 h. The cooled extract was filtered through Whatman No.1 filter paper. It was concentrated by placing in a water bath to remove the complete solvent content. The concentrated extract was stored in an amber-coloured bottle at 4°C for further studies.

Quantification of major classes of compounds revealed from the preliminary analyses such as phenol, flavonoid, terpenoid and alkaloid was done as per the standard protocols described below:

i. Total phenolic estimation

Gallic acid is taken as the standard for phenolic estimation. The standard curve was drawn by taking the concentrations of gallic acid as 20, 40, 60, 80 and 100 µg/mL. From the stock (1 mg/mL), 0.1 mL of sample was taken. Into the sample/standard, 500 µL distilled water is added. It is followed by 125 µL of Folin-Ciocalteau reagent (1N). It was kept for 6 min and after

that 1.25 mL of 7% $NaNO_2$ was added. The final volume was made up to 3 mL with distilled water. The solution was thoroughly mixed by shaking. Incubation was done at room temperature for 90 min. The absorbance was measured at 760 nm against the blank (Oueslati et al. 2012). The total phenolic content is expressed in milligram gallic acid equivalent per gram of dry weight (mg GAE/g DW). All the samples were analysed in triplicates.

ii. Total flavonoid estimation

The aluminium chloride colourimetric method proposed by Oueslati et al. (2012) was employed for the estimation of the total flavonoid content of the plant extracts with slight modifications. Quercetin was used as the standard for estimation. About 0.5 mL (1 mg/mL) of sample and different concentrations of the standard (20, 40, 60, 80 and 100 µg/mL) was mixed with 0.5 mL of distilled water and 300 µL of $NaNO_2$ solution (7%) for 5 min. Into this 300 µL of $AlCl_3$ (10%) solution was added. After 5 min incubation at 25ºC, immediate addition of 0.3 mL 10 % $AlCl_3$ (w/v) was done. The reaction mixture was thoroughly shaken. Into this, 2 mL of 1 M NaOH solution was also poured. The absorbance of the mixture was determined at 510 nm. The calibration curve of quercetin was plotted and the total flavonoid content expressed as milligram quercetin equivalent per gram of dry weight (mg QE/g DW) was determined using a regression equation. Each sample was estimated in triplicates.

iii. Total alkaloid estimation

The total alkaloid content of the three *Strobilanthes* species was examined using the protocol of Shamsa et al. (2008). Caffeine is used as the standard. For the preparation of the standard curve, 20, 40, 60, 80 and 100 µg/mL concentrations were selected. One mL of the extract (1 mg/mL) or standard was dissolved in 1N HCl. Into the sample, 5 mL of phosphate acetate

buffer of pH 4.7 was added. It was followed by the addition of 5 mL of bromocresol (BCG) solution and shaken well. A series of additions from 1, 2, 3 and 4 mL to add a total of 10 mL chloroform was done. It will create two layers. So, the upper BCG layer is pipetted out. The lower chloroform layer containing alkaloids is taken for measuring its absorbance at 470 nm. The calibration curve of caffeine was plotted to generate the regression equation. The total alkaloid content of the extract was calculated and expressed in milligram caffeine equivalent per gram of dry weight (mg CE/g DW). All the samples were analysed in triplicates.

iv. Total terpenoid estimation

Strobilanthes species were screened for their terpenoid content using the method proposed by Ghorai et al. (2012) with some modifications. Linalool was used as the standard for estimation. An aliquot of the sample (0.2 mL) with a concentration of 1 mg/mL was taken. Into these few drops of chloroform and H_2SO_4 was poured. It is then transferred to a colourimetric cuvette. The absorbance was measured at 538 nm against blank i.e., 95% (v/v) methanol. The calibration curve was plotted with concentrations of linalool as 20, 40, 60, 80 and 100 µg/mL. The total terpenoid content of the extract was expressed as milligram linalool equivalent per gram of dry weight (mg LE/g DW). All the samples were analysed in triplicates.

c. Gas Chromatography/Mass Spectrometry (GC/MS) analysis

The volatile components of the extract were evaluated using GC/MS Shimadzu QP-2010 Plus with Thermal Desorption System TD 20, fitted with a 60 m × 0.25 mm × 0.25 m WCOT column coated with diethylene glycol (AB-Innowax 7031428, Japan). The flow rate of the carrier gas Helium was at a rate of 1.21 mL/min. The column pressure was 77.6 kPa and the temperature was maintained at 260ºC. The separation of the component was performed at

a linear temperature program of 70 - 260ºC at 3ºC/min and then held at 260ºC. The time required was 40-50 min. The parameters used for Mass Spectrometry were electron ionisation (EI) voltage 70 eV, peak width 2 s and detector voltage 1.5 V. By comparing the retention indices, the constituents were identified. The MS pattern was checked with pure compounds of known composition and compared with MS fragmentation patterns of National Institute of Standards and Technology (NIST) libraries. Compounds detected are also compared with the previous literature. The quantification of the components was done by calculating GC peak areas.

d. High-Resolution Liquid Chromatography/Mass Spectrometry (HR-LC/MS) analysis

The non-volatile constituents of the extract were screened using HR-LC/MS Q-TOF (Agilent, USA). It is equipped with an electrospray ionization source. The dimension of the column CAPCELL C18, MG-II type was 4.6 mm × 250 mm with a particle size of 5 µm. The gradient flow was at the rate of 0.5 mL/min. The mobile phases used for isocratic elution were 5 to 95% water and 5% acetonitrile. The injection volume was 5 µL, for a full loop injection of 30 min. Using the operating parameters such as nebulizer gas, nitrogen (13 L/min), gas temperature (250ºC) and nozzle voltage (1000 V), eluted compounds were detected.

PHARMACOGNOSTIC PROFILING

The botanical pharmacognosy of powdered plant material can be guaranteed using various methods. In this study, powder microscopic evaluation and ICP-MS analysis were carried out for the selected species of *Strobilanthes*.

a. Powder microscopy

Powder microscopy has strong data support in determining the pharmacological value of a plant. All the three plants under consideration were dried in shade and made into a fine powder using a mortar and pestle. The fine powder is soaked in 4% potassium hydroxide (KOH) overnight. Then they were mounted in a glass slide using glycerine and observed under Nikon ECLIPSE E200 trinocular microscope attached with Zeiss AxioCam ERc 5s digital camera.

b. Inductively Coupled Plasma Mass Spectrometry (ICP-MS) analysis

About 0.2 g of the powdered sample was weighed. It was transferred into a beaker and 15 mL con. HNO_3 was added to it. The beaker was kept closed and heated in a hot plate at a temperature not excluding 120º C. Into this solution, a few drops of H_2O_2 was added and boiled them till forming a clear solution. If the residue still exists, more nitric acid is added. To oxidise the organic contents more drops of H_2O_2 is added to the sample. To get clearer sample Con. H_2SO_4 is poured drop by drop. When the sample gets boiled the temperature can be lowered. When no residue was left, the digested solution is filtered through Whatman No. 1 filter paper and transferred into a 50 mL beaker. The solution is made up to 50 mL using Milli-Q water. This solution was analysed in Agilent 7800 ICP-MS with Integrated Sample Introduction System (ISIS) and SPS 4 autosampler. The injector was a standard torch of 2.5 mm diameter. The instrument uses Ni sampler and Ni skimmer cones.

PHASE III - BIOACTIVITIES

A. Free radical scavenging assays

Extract stock preparation

The antioxidant activity of the methanolic extracts was analysed. 10 mg of the extract was dissolved in 1 mL of DMSO for preparing the stock solution. From the stock, different concentrations such as 125, 250 500, 1000 and 2000 µg/mL were prepared for all the assays except DPPH radical scavenging assay. For the DPPH assay, the concentrations taken were 12.5, 25, 50, 100 and 200 µg/mL. The absorbance itself is considered as a percentage of inhibition in reducing power assay. The inhibition percentage in all the other three assays was calculated using the formula.

$$\text{Inhibition } (\%) = \frac{\text{Ac} - \text{As}}{\text{Ac}} \times 100$$

where Ac and As are the absorbance values of control and sample respectively. Each extract was tested in triplicates and values expressed in mean ± SE.

i. DPPH radical scavenging assay

0.1 mM DPPH was prepared by dissolving 4 mg of 1, 1-diphenyl-2-picryl hydrazyl in 100 mL methanol. Ascorbic acid was taken as the standard (10 mg/mL DMSO). Different concentrations of the extract and standard were made. They were made up to the final volume by adding 20 µL DMSO and 1.48 mL DPPH (Chang et. al., 2001). Distilled water without test compound is taken as the control. After 20 min of incubation in the dark, absorbance was measured at 517 nm. About 3 mL of DPPH was taken as blank in absorbance

reading. The percentage of inhibition was calculated using the above-mentioned formula.

ii. Hydroxyl radical scavenging assay

Different concentrations of the extract and standard were prepared. Gallic acid (10 mg/mL) is taken as the standard in this assay. The reaction mixture consists of 2.8 mM of 2 deoxy 2 ribose, 100 µM of $FeCl_3$, EDTA (100 µM), 1 mM H_2O_2 and 100 µM of ascorbic acid in KH_2PO_4-KOH buffer (20 mM of pH = 7.4) (Caillet et al., 2007). To the prepared concentrations of the extract and standard 50 µL of the reaction mixture was added. The final volume is made up to 1 mL. An equivalent amount of distilled water without a test sample was taken as control. It was followed by incubation at 37°C for one hour. After that 1 mL of TCA (2.8%) and 1 mL of aqueous TBA (1%) were added. It was then incubated for 15 min at 90°C. It was allowed to cool. Absorbance was measured at 532 nm against an appropriate blank solution. The percentage of inhibition was calculated using the above-mentioned formula.

iii. Superoxide free radical scavenging activity

The assay was carried out using ascorbic acid (10 mg/mL) as the standard. Different concentrations of the extract and standard were taken. The reaction mixture consists of 0.05 mL of riboflavin (0.12 mM), 0.2 mL of EDTA solution (0.1 M) and 0.1 mL of Nitro-blue tetrazolium (NBT) solution (1.5 mM). The reaction mixture was diluted up to 2.64 mL using phosphate buffer (0.067 M). Control without the test compound but an equal amount of distilled water was taken. It was incubated in fluorescent light for 5 min and absorbance was measured at 560 nm. The absorbance was also measured at the same wavelength after 30 min of illumination. The change in OD was

calculated (Valentão et al., 2002). The percentage of inhibition was calculated using the above-mentioned formula.

iv. Reducing power activity

Quercetin (10 mg/mL of DMSO) was used as the standard. To the different concentrations of the extract and standard, 2.5 mL of phosphate buffer (200 mM, pH = 6.6) and 2.5 mL of potassium ferric cyanide (1%) was added. The reaction mixture was boiled for 20 min at 50°C (Yen & Duh, 1993). Control without the test compound but an equal amount of distilled water was taken. After incubation 2.5 mL of TCA (10%) was added. It was centrifuged at 650 g for 10 min. The upper layer was mixed with an equal amount of distilled water. It is followed by the addition of 1 mL of 0.1% ferric chloride. The absorbance was read at 700 nm.

B. Cytotoxicity by *Allium cepa* assay

Rooted *A. cepa* bulbs were used for the evaluation of cytotoxicity. Healthy bulbs with germinated roots were collected at peak mitotic activity (9 – 10 am). They are treated with different concentrations of shoot extract (100, 75 and 50 µg/mL) for 24 h. shoot extracts were taken in a small vial, and the bulbs were kept at the rim of the vials, so that only the roots were exposed to the extract solution. Roots were excised from the bulbs after treatment. They were fixed in modified Carnoy's fluid (**Appendix 1**) for 1 h. It is followed by hydrolysis using 1N HCl for a few minutes. Staining was done for 3 h in acetocarmine (**Appendix 4**). Hydrogen peroxide (CAS No: 7722-84-1, µg/mL) and distilled water were taken as the positive and negative control for the assay respectively. Then they were destained and mounted in 45% acetic acid. Mitotic squash preparations were done after following a standard protocol (Sharma & Sharma, 1990). Images were acquired and cytotoxic parameters were calculated using the following formulae.

$$\text{Mitotic index} = \frac{\text{Number of dividing cells}}{\text{Total number of cells}} \times 100$$

$$\text{Abnormality percentage} = \frac{\text{Number of aberrant cells}}{\text{Total number of cells}} \times 100$$

C. Antiproliferative efficacy of *Strobilanthes*

For the antiproliferative studies, two cell lines were selected. They were Human Colorectal Adenocarcinoma (DLD1) and normal fibroblast cells (L929) (**Plate 2**).

i. *In vitro* anticancer activity on DLD1 cell line using MTT assay

The DLD1 cells (Human Colorectal Adenocarcinoma) were procured from National Centre for Cell Sciences (NCCS), Pune, India and maintained in DMEM (**Appendix 5**) (Sigma-Aldrich, USA) medium. It was supplemented with 10% FBS, L-glutamine, sodium bicarbonate (Merck, Germany) and an antibiotic solution containing: Penicillin (100 µg/mL), Streptomycin (100 µg/mL), and Amphoteracin B (2.5 µg/mL). It was kept at 37ºC in a humidified 5% CO_2 incubator. Using an inverted phase-contrast microscope direct observations of cells were done, followed by an MTT assay.

❖ **Cell seeding in 96 well plate**

Two days old confluent monolayer of cells was trypsinized and the cells were suspended in a 10% growth medium. The 100 µL cell suspension was seeded in a culture plate and incubated at 37ºC in a humidified 5% CO_2 incubator.

❖ **Extract preparation and anticancer evaluation**

One milligram of the extract was weighed and dissolved in 1 mL Dulbecco's modified Eagles medium (DMEM) using a cyclomixer. The

sample solution was filtered through a Millipore syringe filter to ensure sterility. Different concentrations of the extract (100, 50, 25, 12.5, 6.25 µg in 500 µL of 5% DMEM) were prepared. From this 100 µL of each concentration of the prepared extract was added in triplicates to the respective wells. In the same condition, untreated controls were also maintained.

❖ **Direct microscopic observation of anticancer activity**

Microscopic observation of anticancer effect on DLD1 cell lines was done after 24 h of treatment with the help of an inverted phase-contrast tissue culture microscope (Olympus CKX41 with Optika Pro5 CCD camera). The different types of morphological alterations such as cell shrinkage, vacuolization and cytoplasmic granulation *etc.,* indicates the cytotoxic effect.

❖ **Anticancer activity by MTT assay**

MTT (15 mg) was completely dissolved in a 3 mL phosphate buffer solution and sterilized by filter sterilization. After 24 h of the incubation period, the sample content in wells was removed and 30 µL of prepared MTT solution was added to all test and control wells. The plate was gently shaken well and incubated at 37ºC in a humidified 5% CO_2 incubator for 4 h. Then the supernatant was removed and 100 µL of MTT solubilization solution (Dimethyl sulphoxide, DMSO – Sigma-Aldrich, USA) was added and mixed gently by pipetting up and down in order to solubilize the formazan crystals. The absorbance values were measured by using a microplate reader at a wavelength of 540 nm (Talarico et al., 2004). The percentage of viability was calculated using the formula.

$$\text{Viability (\%)} = \frac{\text{Mean OD of samples}}{\text{Mean OD of control}} \times 100$$

ii. *In vitro* cytotoxic effect on normal cell line by MTT assay

The L929 cell line (normal cell line) was procured from National Centre for Cell Sciences (NCCS), Pune, India and maintained in DMEM. It was supplemented with 10% FBS, L-glutamine, sodium bicarbonate (Merck, Germany) and an antibiotic solution containing: Penicillin (100 µg/mL), Streptomycin (100 µg/mL), and Amphoteracin B (2.5 µg/mL). It was kept at 37ºC in an incubator (humidified 5% CO_2 - NBS Eppendorf, Germany). All the extracts were analysed in normal cell lines to check the cytotoxicity. The concentrations selected were 100, 50, 25, 12.5 and 6.25 µg/mL of 5% DMEM. The cell viability was evaluated by direct observation of cells by using an inverted phase-contrast microscope, followed by the MTT assay method as described earlier.

iii. Apoptosis detection using acridine orange and ethidium bromide (AO-EtBr) double staining

Acridine Orange (AO) and Ethidium Bromide (EtBr) are DNA binding fluorescent dyes (Sima-Aldrich, USA) (**Appendix 6**). They were used in the morphological detection of apoptotic and necrotic cells (Zhang et al., 1998). The non-viable cells take up EtBr dye which is intercalated into DNA and emit red fluorescence. But the viable cells take up the AO stain and emit green fluorescence when intercalated into DNA. This gives a morphological differentiation to viable and non-viable cells.

After proper culturing and seeding of DLD1 cell lines, LC_{50} concentrations of all three samples were added separately and kept for 24 h. The LC_{50} concentrations were 185.09, 115.64 and 111.999 µg/mL of *S. anamallaica, S. hamiltoniana* and *S. virendrakumarana* respectively. It is followed by a wash in cold phosphate buffer solution (PBS) and then stained with a mixture of AO (100 µg/mL) and EtBr (100 µg/mL) at room

temperature for 10 min. The stained cells were washed twice with 1X PBS and observed in a blue filter of a fluorescent microscope (Olympus CKX41 with Optika Pro5 CCD camera).

A total of four categories can be visualized in the field. The normal green nucleated ones are living cells, a bright green nucleus with fragmented or condensed chromatin are early apoptotic cells, orange nuclei with chromatin condensation or fragmentation are late apoptotic ones and uniformly orange stained cells are necrotic ones.

iv. Cell cycle analysis using cell cycle kit by flow cytometry

The best method to analyse the health of a cell is by directly measuring the DNA synthesis. The standard ethanol fixation and detergent permeabilization is the principle behind the MUSE cell cycle kit. It has the ability to get access to DNA while the cell cycle is in progress. DLD-1 cells were cultured as per standard procedures described earlier. They were treated with 111.999 µg/mL *S. virendrakumarana* (LC_{50}) for 24 h. The cell sample was transferred to a 50 mL conical flask or 12 × 75 mm polystyrene tube. In a tube, the recommended number for fixation of a cell line is 1×10^6 cells. The tube containing the sample is centrifuged at 3000 rpm for 5 min. The cell pellet can be seen at the bottom. The supernatant is removed without disturbing the pellet.

PBS solution was added to each tube which is about 1 mL of PBS per 1×10^6 cells and the contents were mixed by pipetting several times or gently vortexing. Again, it was centrifuged at 3000 rpm for 5 min. The supernatant was removed and resuspended the pellet in PBS solution by repeated pipetting several times or gently vortexing. The resuspended cells were added dropwise into the tube containing 1 mL of ice-cold 70% ethanol while vortexing at medium speed. It was stored at -20ºC overnight.

Overnight incubation is followed by centrifuging the sample at 3000 rpm for 5 min at room temperature. The supernatant was removed and about 250 µL PBS was added to the pellet. Repeat the centrifugation at the same revolution and time. It results in the formation of pellets. The supernatant was removed and 250 µL of cell cycle reagent was added. It is followed by incubation in dark for 30 min (which is light sensitive). After the incubation, the samples were analysed in a flow cytometer. The gating was performed with reference to untreated control cells.

v. Gene expression study using RT-qPCR

❖ **Isolation of total RNA**

The total isolation kit was used for the isolation of total RNA (Invitrogen – Product code 10296010). To release the RNA, cell has to be disrupted using the addition of TRIzol solution. During the chloroform extraction followed by the centrifugation, RNA will be in the aqueous phase. Whereas proteins are in the interphase and organic phase. On addition of isopropanol, RNA gets precipitated as a white pellet on the side and the bottom of the tube. DLD-1 (Human colon adenocarcinoma) cell line was cultured and maintained as per the standard procedure described previously. After attaining 70% confluency of cells in 6 well plates, the cells were treated with LC_{50} (111.999µg/mL) concentration of the extract of *S. virendrakumarana*. A separate set of untreated control cells were also incubated at 37^0 C for 24 hours in a CO_2 incubator.

After incubation, the DMEM medium was removed and 200 µL of TRIzol reagent was added to the culture plate, followed by incubation for 5 min. It was then transferred to a fresh sterile Eppendorf tube. It is followed by the addition of 200 µL of chloroform and vigorously shaken for 15 seconds and incubated for 2-3 min at room temperature, followed by centrifugation at

14000 rpm for 15 min at 4^0 C. The supernatant in the tube was discarded and the pellet thus obtained was washed with 200µL of 75% ethanol (Merck). Again, it was centrifuged at 14000 rpm for 5 min at 4^0 C in a cooling centrifuge. Lastly, the RNA pellet was dried and suspended in Tris-EDTA buffer (**Appendix 7**).

❖ **Synthesis of cDNA**

With the help of a TRI reagent (Sigma) total RNA was extracted. The purity and the concentration of total RNA was determined. The template complementary DNA was synthesized using the cDNA preparation kit (Thermo Scientific, Product code- AB1453A, Verso cDNA Synthesis kit). Into this, about 4 µL of 5X cDNA synthesis buffer, 2 µL of dNTP mix, 1 µL of anchored oligo dT, 1 µL of RT Enhancer, 1 µL of Verso Enzyme Mix and 5 µL of RNA template (1ng of total RNA) were added in an RNAse free tube. The total reaction volume was made up to 20 µL with the addition of sterile distilled water. It was mixed gently by pipetting up and down. The thermal cycler (Eppendorf Master Cycler) was programmed to undergo cDNA synthesis. The following cycling conditions were employed, 30 min at 42ºC and 2 min at 95ºC.

Step	Temperature (ºC)	Time (min.)	Number of cycles
cDNA synthesis	42	30	1
Inactivation	95	2	1

❖ **Gene expression analysis using RT-qPCR**

Real-Time qRT-PCR analysis was carried out using SYBR Green Master Mix (Applied Biosystem, Life technologies). All reactions were performed in triplicates and data were analysed according to ΔΔCt method

using Light Cycler 96 SW 1.1 Software. The steps involved, time taken, temperature and primer sequences used were summarized and shown below.

Steps	Time required	Temperature
Initial activation step	2 min	95^{o}C
3 step cycling:		
Denaturation	10 seconds	94^{o}C
Annealing	1 min	55^{o}C
Extension	1 min/kb	72^{o}C
Number of cycles	40 cycles	68^{o}C
End of PCR cycling	Indefinite	4^{o}C

The primer sequences used:

Human GAPDH forward	5'-ACTCAGAAGACTGTGGATGG-3'
Human GAPDH backward	3'-GTCATCATACTTGGCAGGTT-5'
Human Bcl-2 forward	5'-CCTGTGGATGACTGAGTA-3'
Human Bcl-2 backward	3'-GAGACAGCCAGGAGAAATCA-5'
Caspase-3 forward	5'-TTCAGAGGGGATCGTTGTAGAAGTC-3'
Caspase-3 backward	3'-CAAGCTTGTCGGCATACTGTTTCAG-5'

❖ **Agarose gel electrophoresis**

The amplified DNA fragments can be separated and visualised using agarose gel electrophoresis. With the help of an electrolyte solution (buffer) an electric field is generated. The separation of fragments is based on charge and size and they move through agarose gel matrix, when subjected to an electric field. The agarose dissolves when boiled in an aqueous buffer, and upon cooling solidifies to a gel. About 1.5% agarose gel was prepared in 1x TAE buffer and melted in hot water bath at 90ºC. The melted agar was cooled down to 45^{0}C. Into this 6 µL ethidium bromide (10 mg/mL) was added and poured in to a gel casting apparatus with the gel comb. When the gel settles,

the comb was removed. The electrophoresis buffer was poured into the gel tank and the platform with the gel was placed in it so as to immerse the gel. The sample was loaded into the gel and run at 50 V for 30 min. with the help of a gel documentation system (E gel imager, Invitrogen) the stained gel was visualised.

STATISTICAL ANALYSIS

The data obtained throughout the work were subjected to statistical analysis using IBM SPSS Statistics Version 20. The data obtained were analysed in One-Way ANOVA followed by the Duncans Multiple Range Test (DMRT) to confirm the variability of data and validity of results. All the values were expressed in mean ± standard error. The statistical significance was determined with $p<0.05$, which is considered significant.

RESULTS

PHASE I - CYTOGENETICAL STUDIES

Strobilanthes species subjected to karyomorphometrical characterization have shown morphologically distinguishable karyotypes of different ploidy levels within a meristematic tissue which can be called mixoploidy or polysomaty. Mixoploidy is the presence of cells with different chromosome numbers and their coexistence in a single tissue of the organism. The mixoploid condition was reported for the first time in the genus *Strobilanthes.* The basic chromosome number in all the species were found to be x = 10. The number of different cytotypes ascertained in *S. anamallaica, S. virendrakumarana* and *S. hamiltoniana* were three, four and two respectively. Among the chromosome pairs, 4 chromosomes were found to be having secondary constrictions in somatic cells of all the species. The number of chromosomes with secondary constrictions increases by one pair as the ploidy increases.

In *S. anamallaica*, the three different cytotypes were observed with karyotype formulae as 2 nsm (-) + 8 nm, 2 M + 18 nm and 30 nm. All the chromosomes were having more or less equal arms pointing to the nearly median or sub-median centromeres (**Tables 5, 6, 7**). In the somatic variant cell with 10 chromosomes (**Plates 3, 4**), 2 chromosomes were found to be having secondary constrictions. The total somatic chromosome (2n = 20) length was 18.19 µm with the length of chromosomes ranging from 2.87-1.23 µm (**Plates 5, 6**). Apart from this, hypoploid (10 chromosomes) and hyperploid (30 chromosomes) somatic variants were present resulting in mixoploidy. In the diploid somatic chromosome cell, all the chromosomes were nearly median except one pair which was with median centromere. The complete 15 pairs of nearly median chromosomes in hyperploid cells revealed

the uniformity in the karyotype (**Plates 7, 8**). The range of chromosome length varies in each ploidy level resulting in variation in average chromosome length. The highest recorded value for As K% and A2 was high in cytotype with 30 chromosomes. But the TF%, Syi index and DI were high in the diploid cytotype with 2n = 20. The various asymmetric indices were also tabulated (**Table 8**).

The annually flowering garden plant, *S. hamiltoniana* have shown only two ploidy levels. The karyotype formulae of the somatic variant cytotype with 10 and the diploid cytotype with 20 chromosomes were 10 nm (**Plates 9, 10**) and 20 nm respectively. The species has nearly median chromosome pairs in both hypoploid and diploid cells (**Tables 9, 10**). The chromosome length was 9.88 and 18.07 in the cells with 2n = 10 and 2n = 20 respectively (**Plates 11, 12**). The chromosome length within the species ranges between 1.18 – 2.8 µm with an average chromosome length below 2 µm. The DI and As K% was found to be high in 2n = 20 but the Syi-Rec indices and TF% were high in cytotype with 10 chromosomes. The A2 index was almost the same in both ploidies (**Table 11**). The intrachromosomal asymmetry index was 0.14 and 0.21 in the variant and normal cytotype respectively.

The karyotype analysis of *S. virendrakumarana* revealed the presence of B-chromosomes (**Tables 12, 13, 14, 15**). The basic set of chromosomes in the plant was found to be x = 10 (**Plates 13, 14**) with the diploid karyotype number of 2n = 20 + 0-1B (**Plate 15, 16**). One to two B chromosomes were observed in different ploidy levels of the plant. Both hypoploid (10 + 0-1B) and hyperploid cells (30 + 0-2B, 40 + 0-2B) were scored in the same meristematic tissue (**Plates 17, 18, 19, 20**). The diploid karyotype formula of the plant was 2 nsm (-) + 18 nm + 0-1 fragment with a total chromosome length of 45.34 µm. The longest chromosome was 2.95 µm whereas the smallest was of length 1.34 µm. The karyotype formulae of the variants with

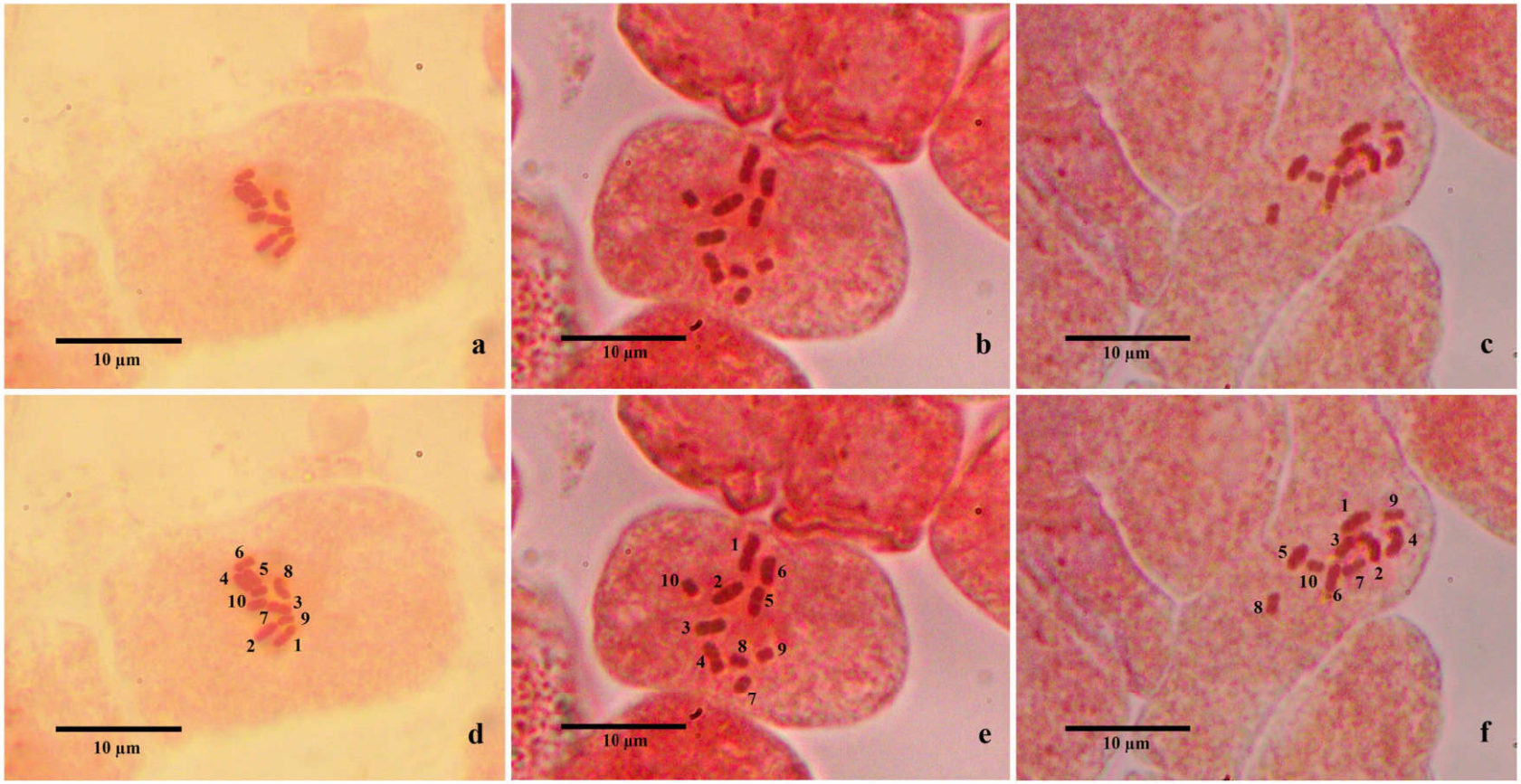


Plate 3: Somatic mitotic metaphase plates of *Strobilanthes anamallaica* with a variant chromosome number of 10, a-c. Cells at mitotic metaphase; d-f. Karyotype with 10 chromosomes

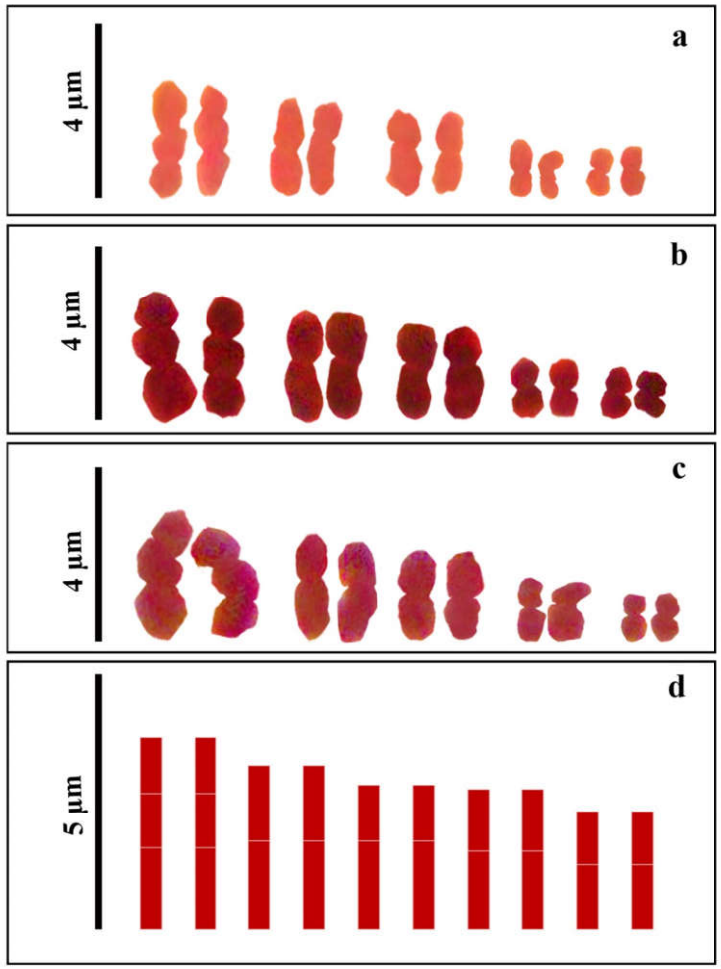


Plate 4: Mitotic variant chromosome complement images of *Strobilanthes anamallaica* with 10 chromosomes 2n = 10, a-c. Karyograms; d. Idiogram

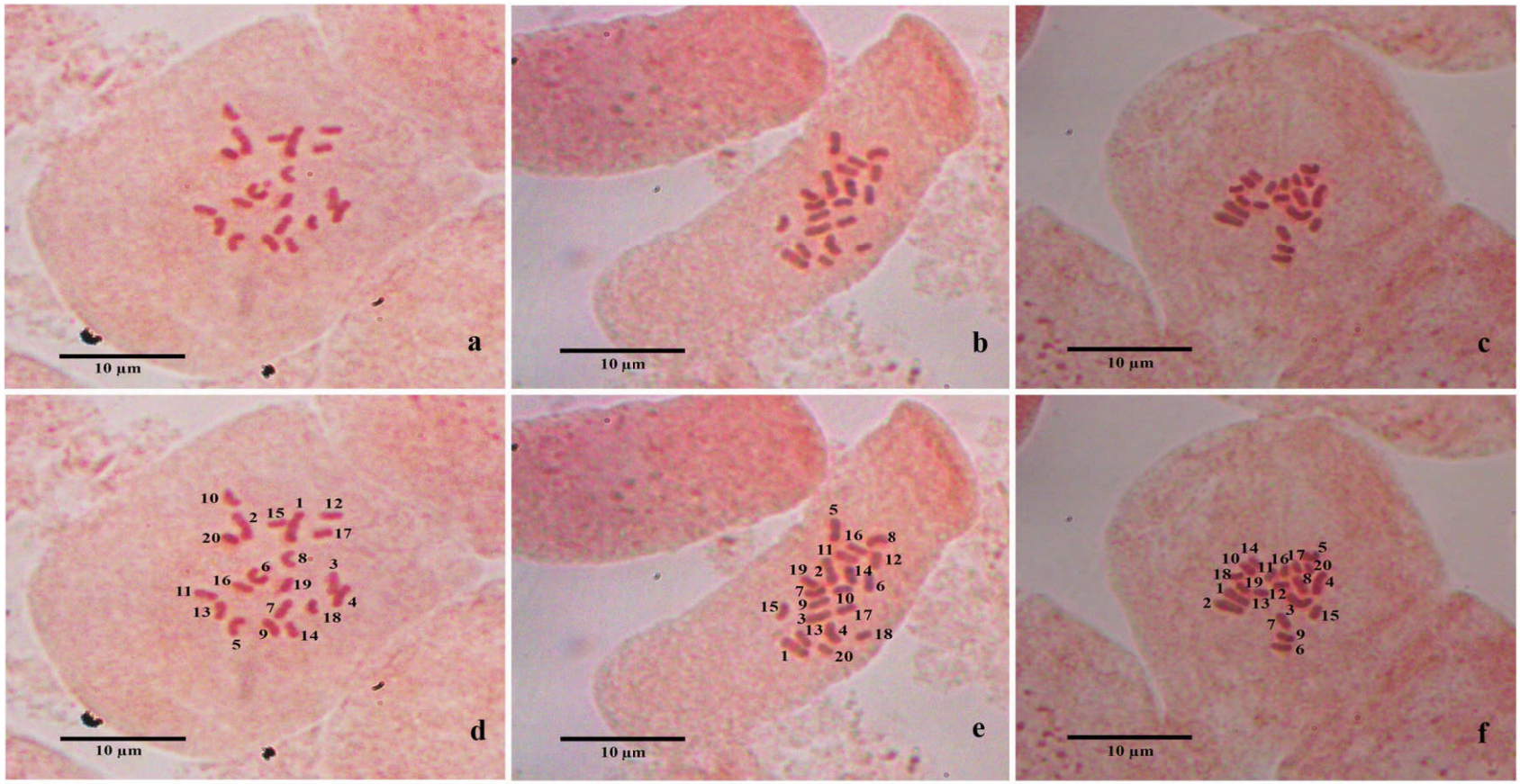


Plate 5: Somatic mitotic metaphase plates of *Strobilanthes anamallaica* with 2n = 20, a-c. Cells at mitotic metaphase; d-f. Karyotype with 20 chromosomes

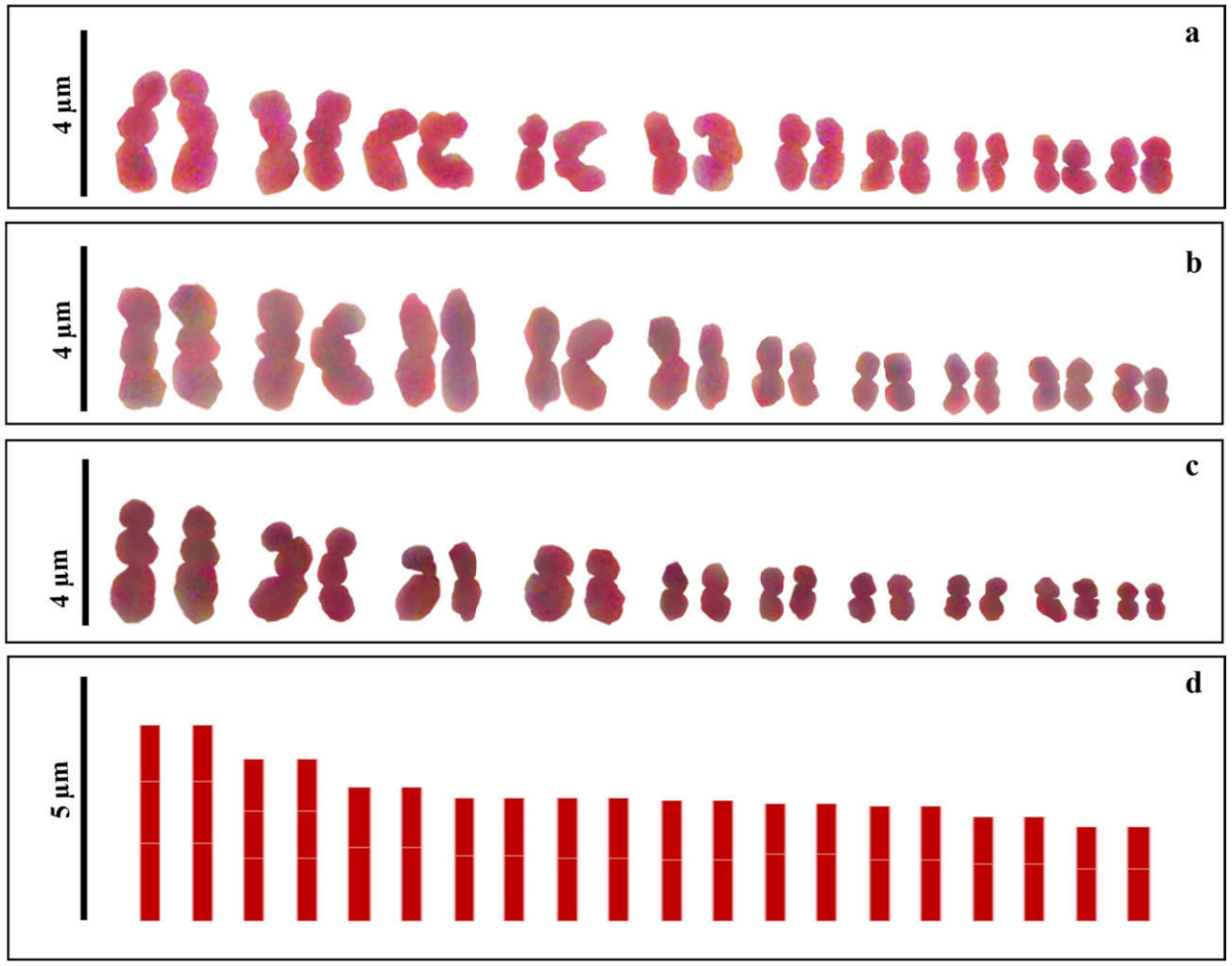


Plate 6: Mitotic diploid chromosome complement images of *Strobilanthes anamallaica* with 2n = 20, a-c. Karyograms; d. Idiogram

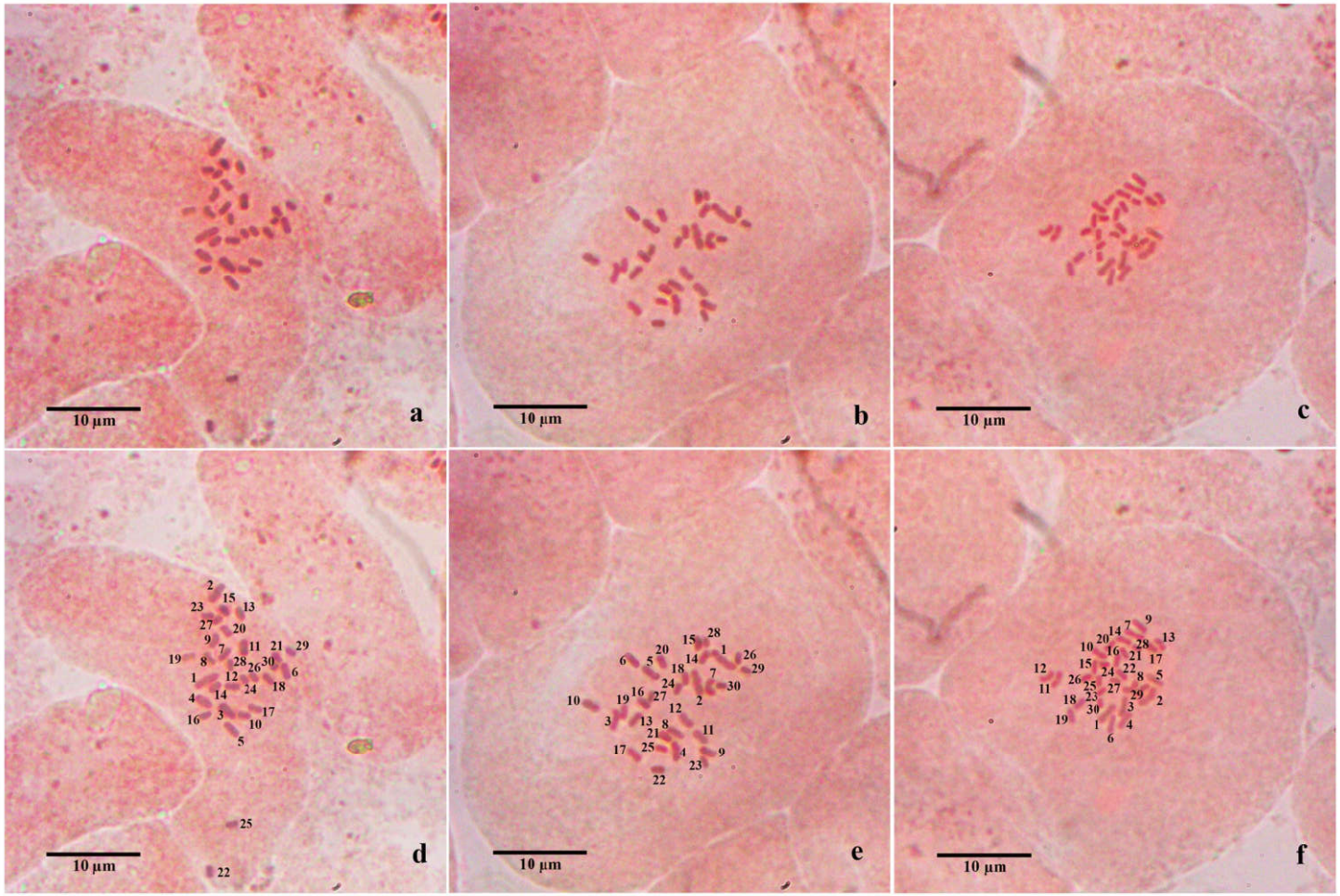


Plate 7: Somatic mitotic metaphase plates of *Strobilanthes anamallaica* with a variant chromosome number of 30, a-c. Cells at mitotic metaphase; d-f. Karyotype with 30 chromosomes

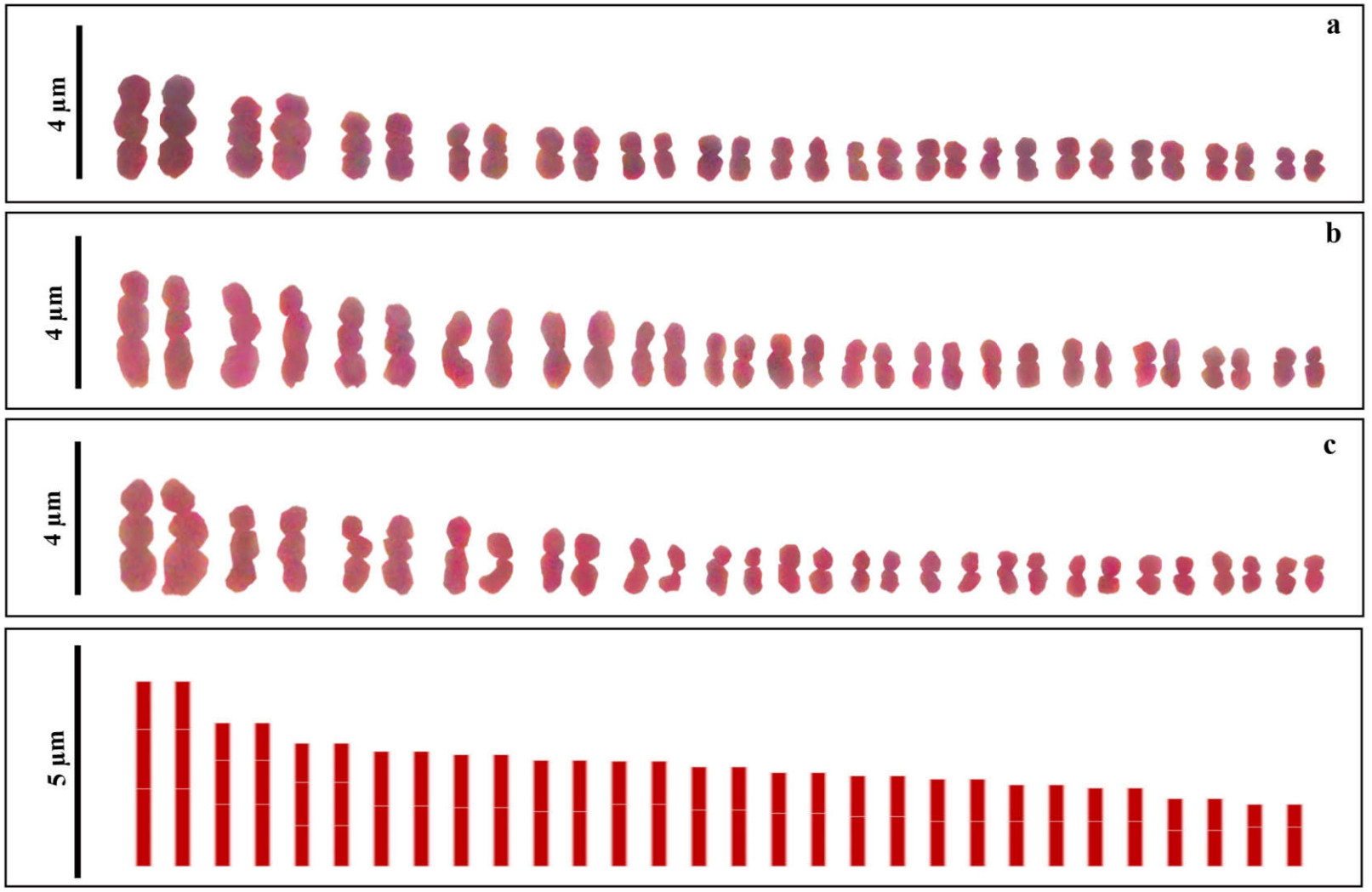


Plate 8: Mitotic variant chromosome complement images of *Strobilanthes anamallaica* with 30 chromosomes, a-c. Karyograms; d. Idiogram

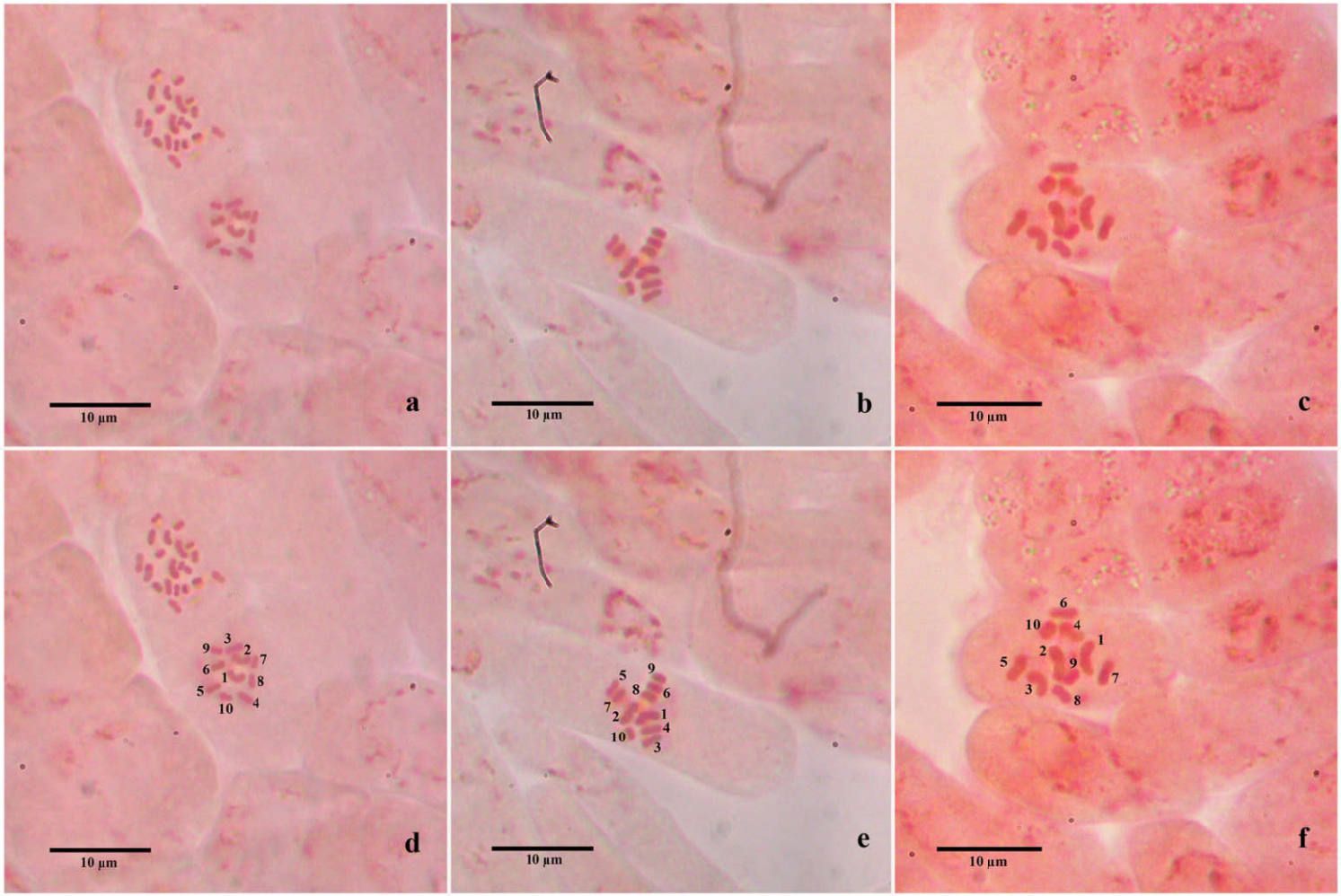


Plate 9: Somatic mitotic metaphase plates of *Strobilanthes hamiltoniana* with a variant chromosome number of 10, a-c. Cells at mitotic metaphase; d-f. Karyotype with 10 chromosomes

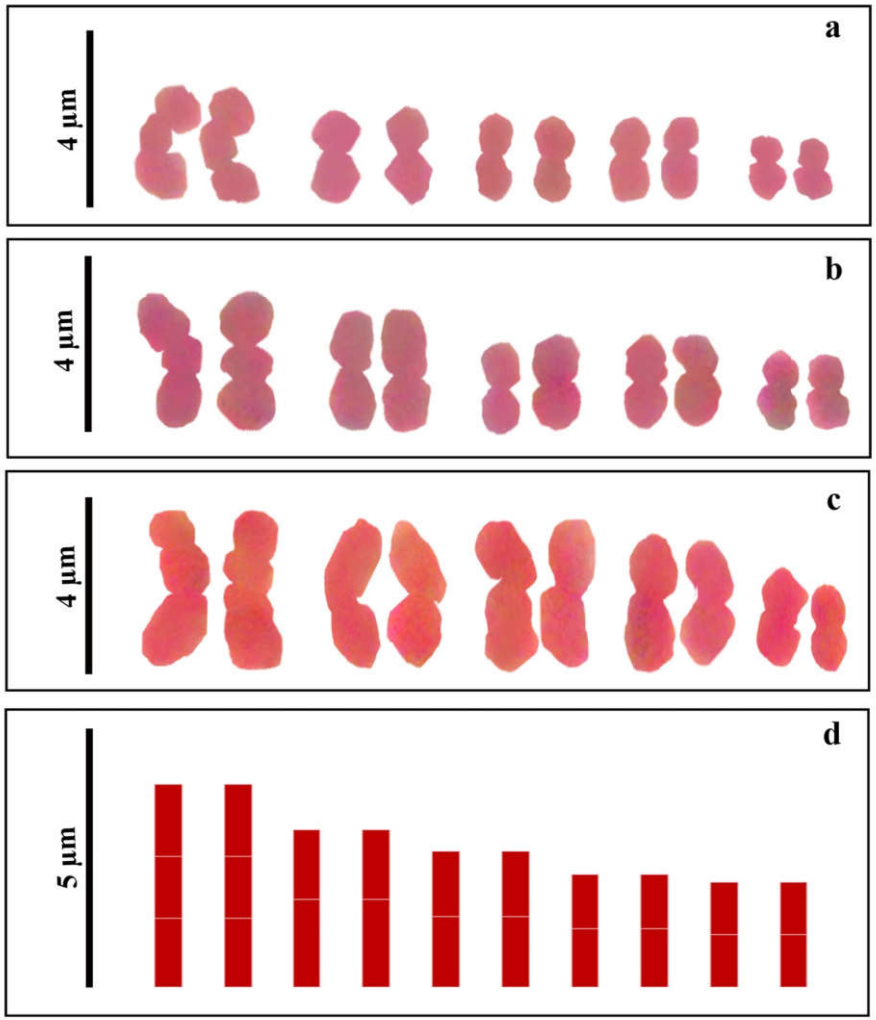


Plate 10: Mitotic variant chromosome complement images of *Strobilanthes hamiltoniana* with 10 chromosomes, a-c. Karyograms; d. Idiogram

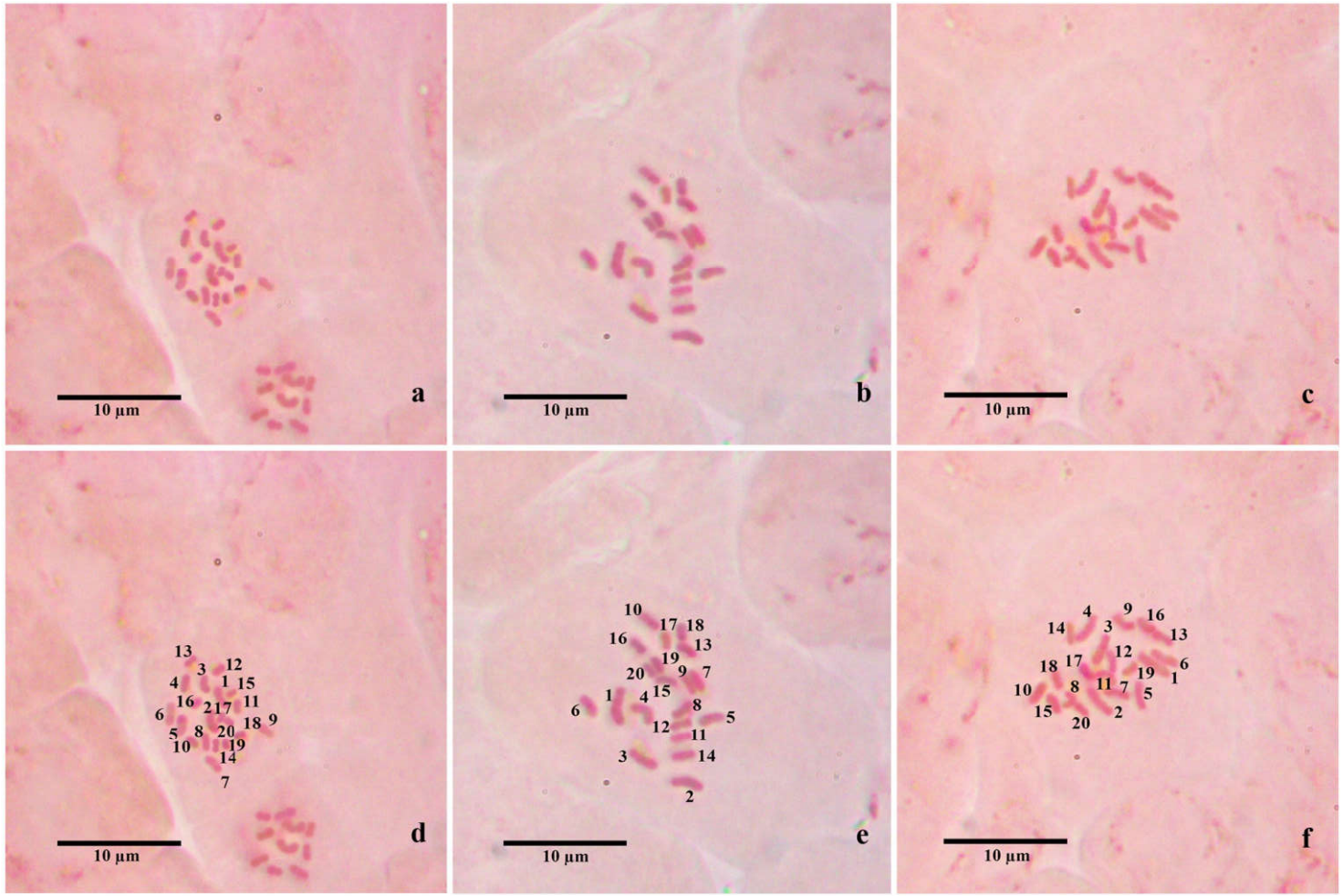


Plate 11: Somatic mitotic metaphase plates of *Strobilanthes hamiltoniana* with 2n = 20, a-c. Cells at mitotic metaphase; d-f. Karyotype with 20 chromosomes

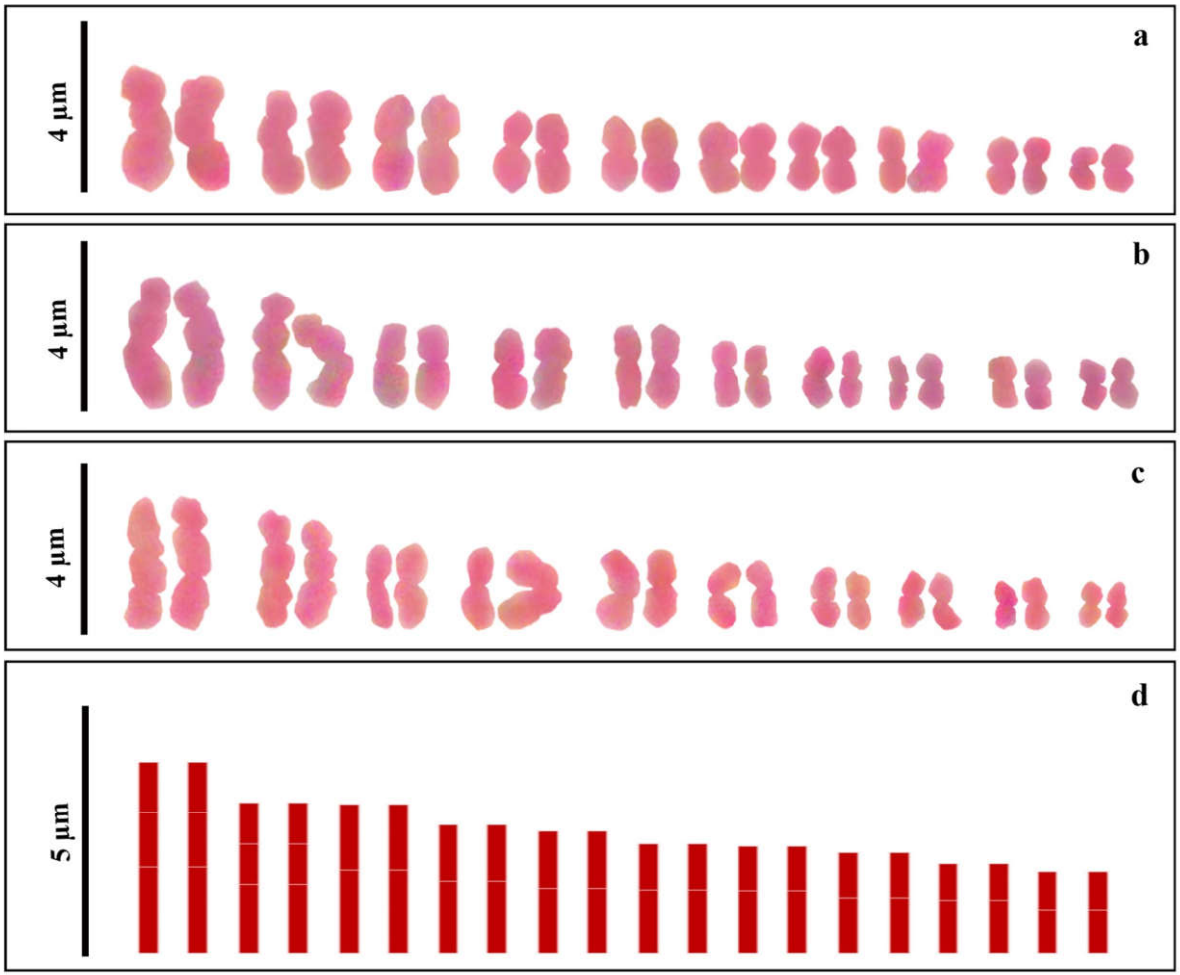


Plate 12: Mitotic diploid chromosome complement images of *Strobilanthes hamiltoniana* with 2n = 20, a-c. Karyograms; d. Idiogram

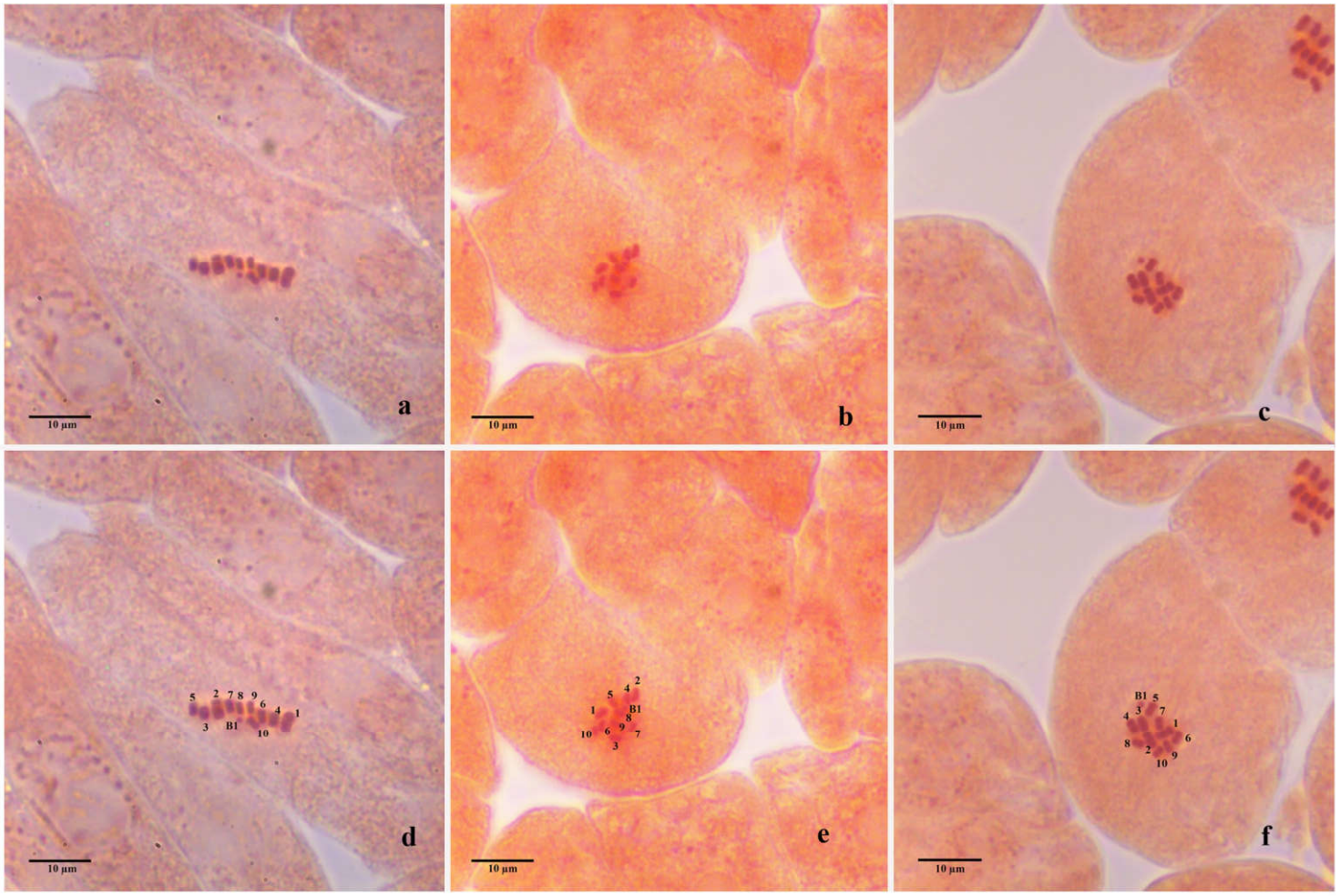


Plate 13: Somatic metaphase chromosome plates of *Strobilanthes virendrakumarana* with a variant chromosome number of 10+0-1B, a-c. Cells at mitotic metaphase; d-f. Karyotype with 10 chromosomes and 0-1B chromosome

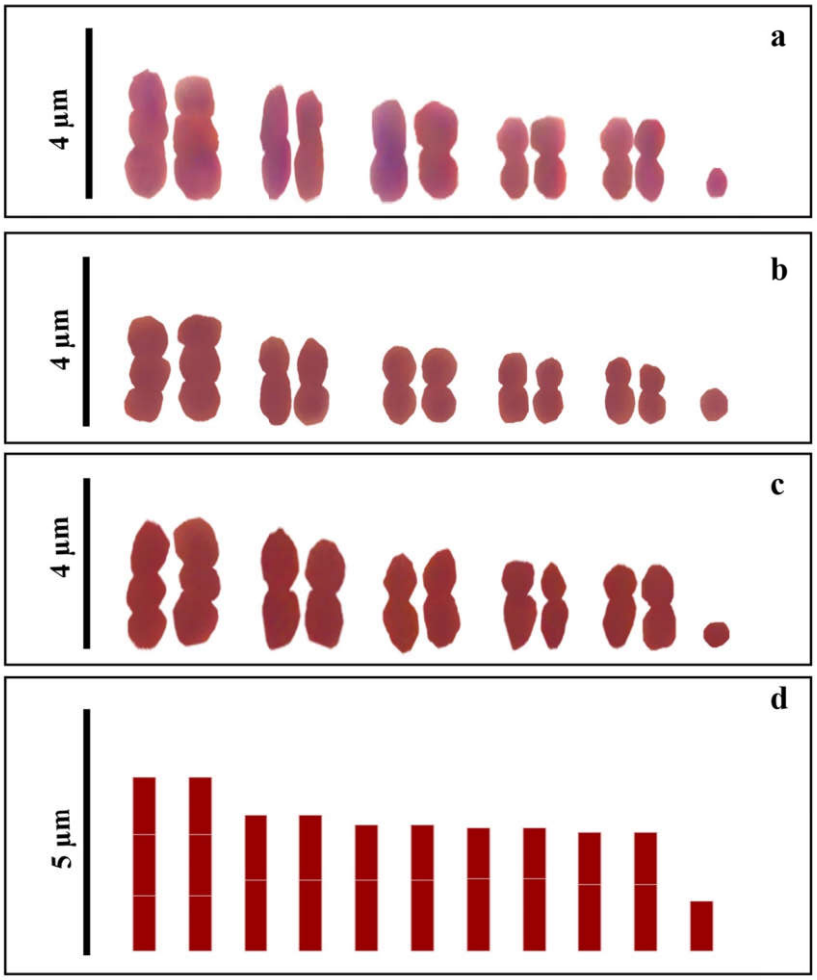


Plate 14: Mitotic variant chromosome complement images of *Strobilanthes virendrakumarana* with 10 chromosomes and 0-1B chromosome, a-c. Karyograms; d. Idiogram

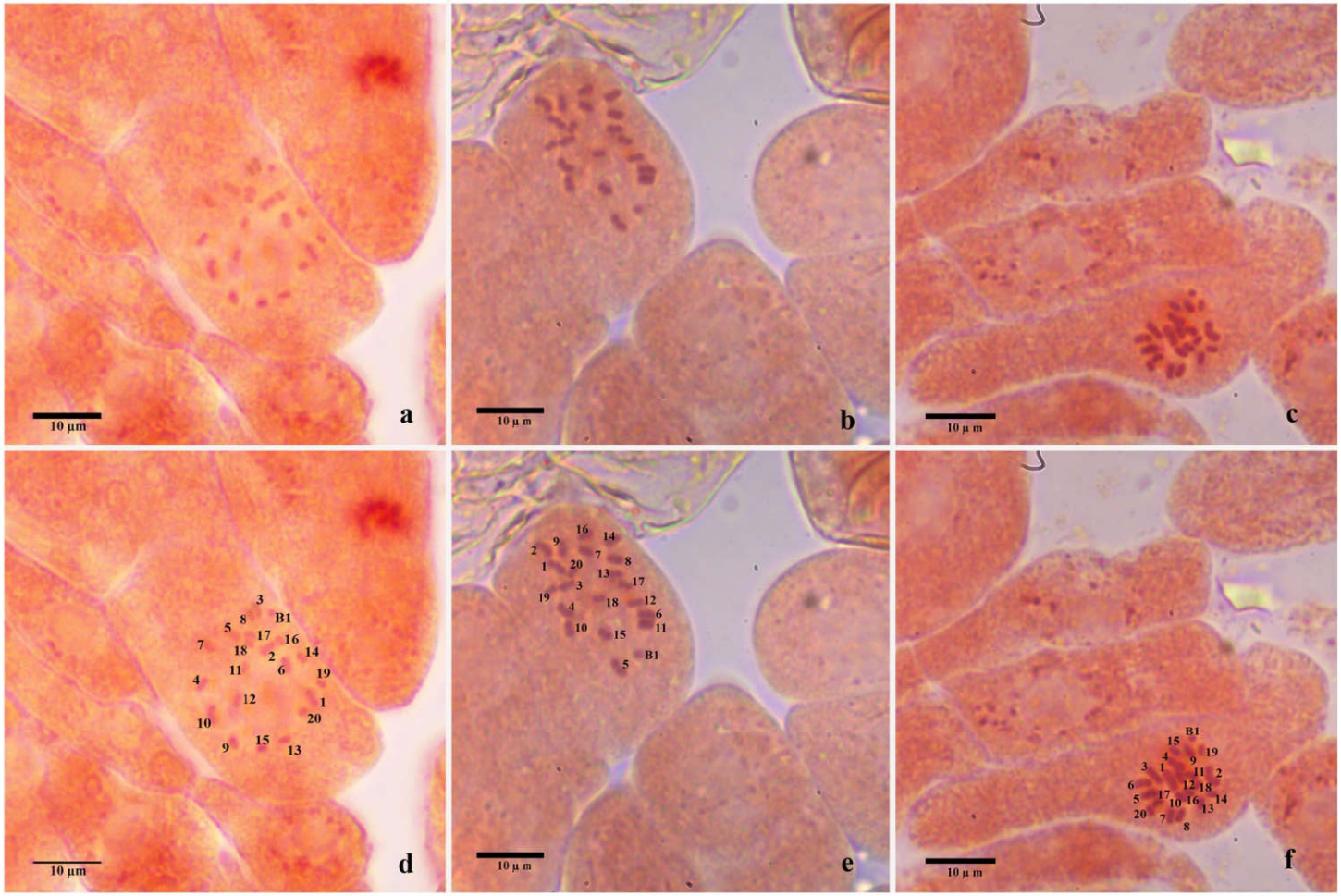


Plate 15: Somatic mitotic metaphase plates of *Strobilanthes virendrakumarana* with 2n = 20+0-1B, a-c. Cells at mitotic metaphase; d-f. Karyotype with 20 chromosomes and 0-1 B chromosome

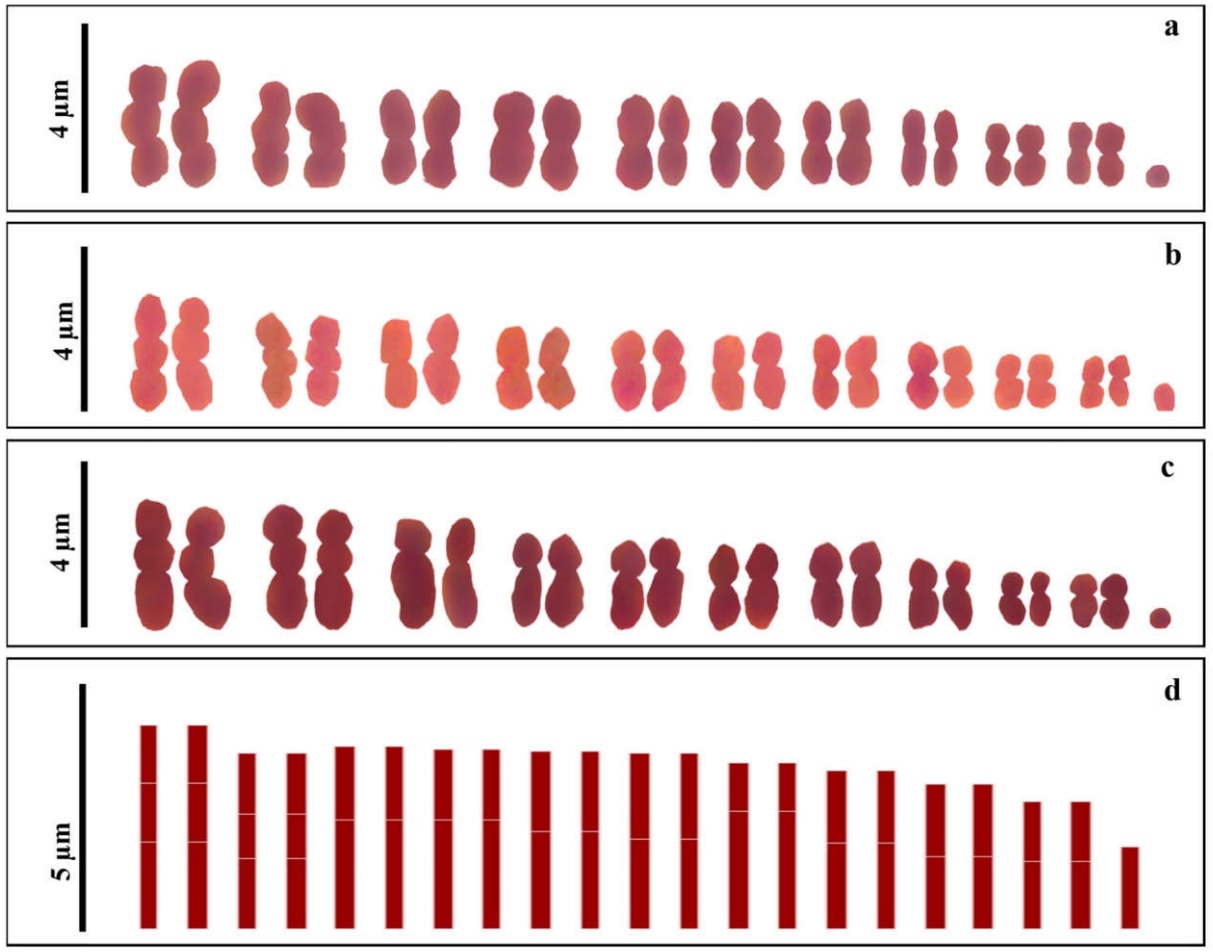


Plate 16: Mitotic diploid chromosome complement images of *Strobilanthes virendrakumarana* with 2n = 20+0-1B, a-c. Karyograms; d. Idiogram

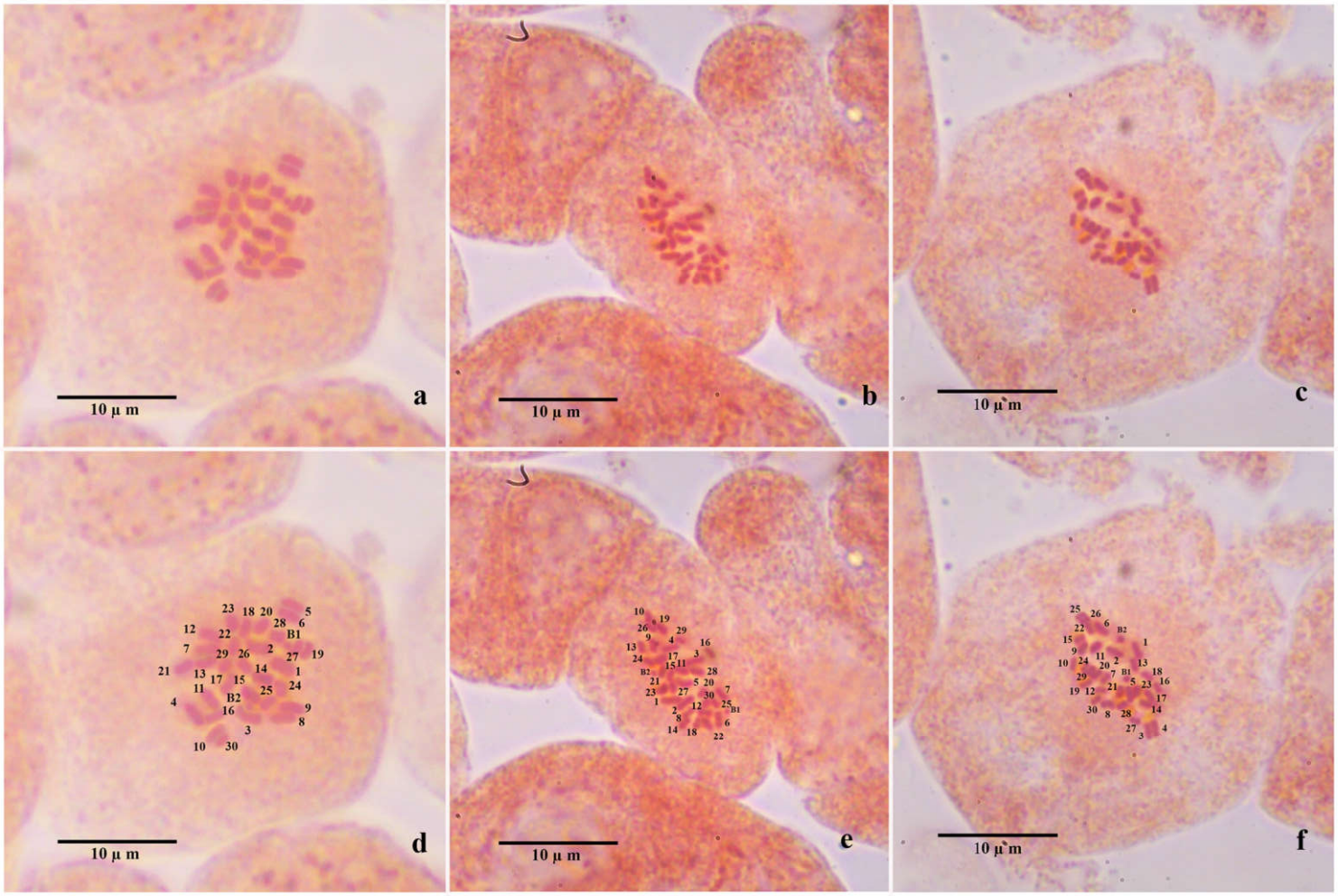


Plate 17: Somatic mitotic metaphase plates of *Strobilanthes virendrakumarana* with a variant chromosome number of 2n=30+0-2B, a-c. Cells at mitotic metaphase; d-f. Karyotype with 30 chromosomes and 0-2 B chromosomes

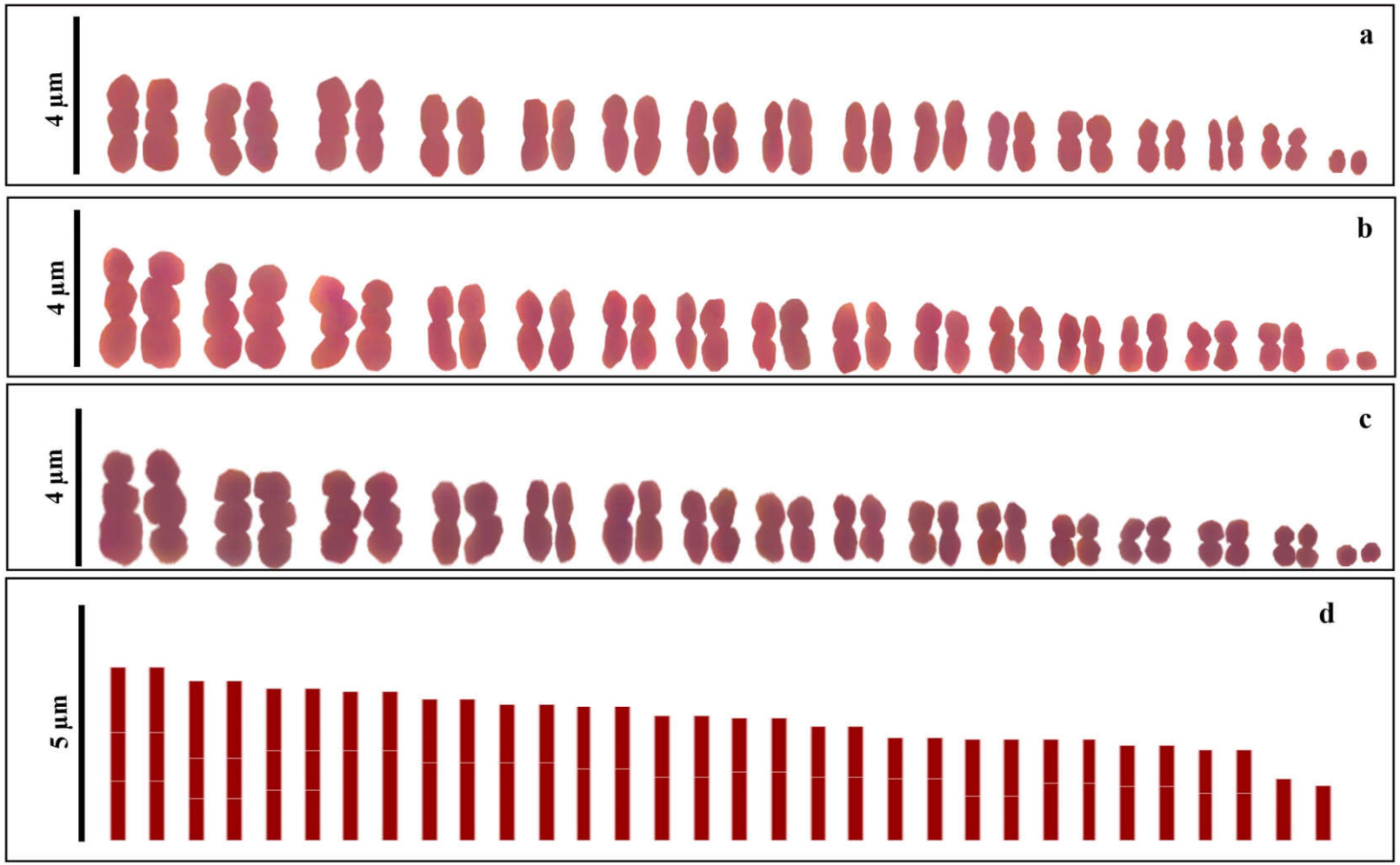


Plate 18: Mitotic variant chromosome complement images of *Strobilanthes virendrakumarana* with 30 chromosomes and 0-2B chromosomes, a-c. Karyograms; d. Idiogram

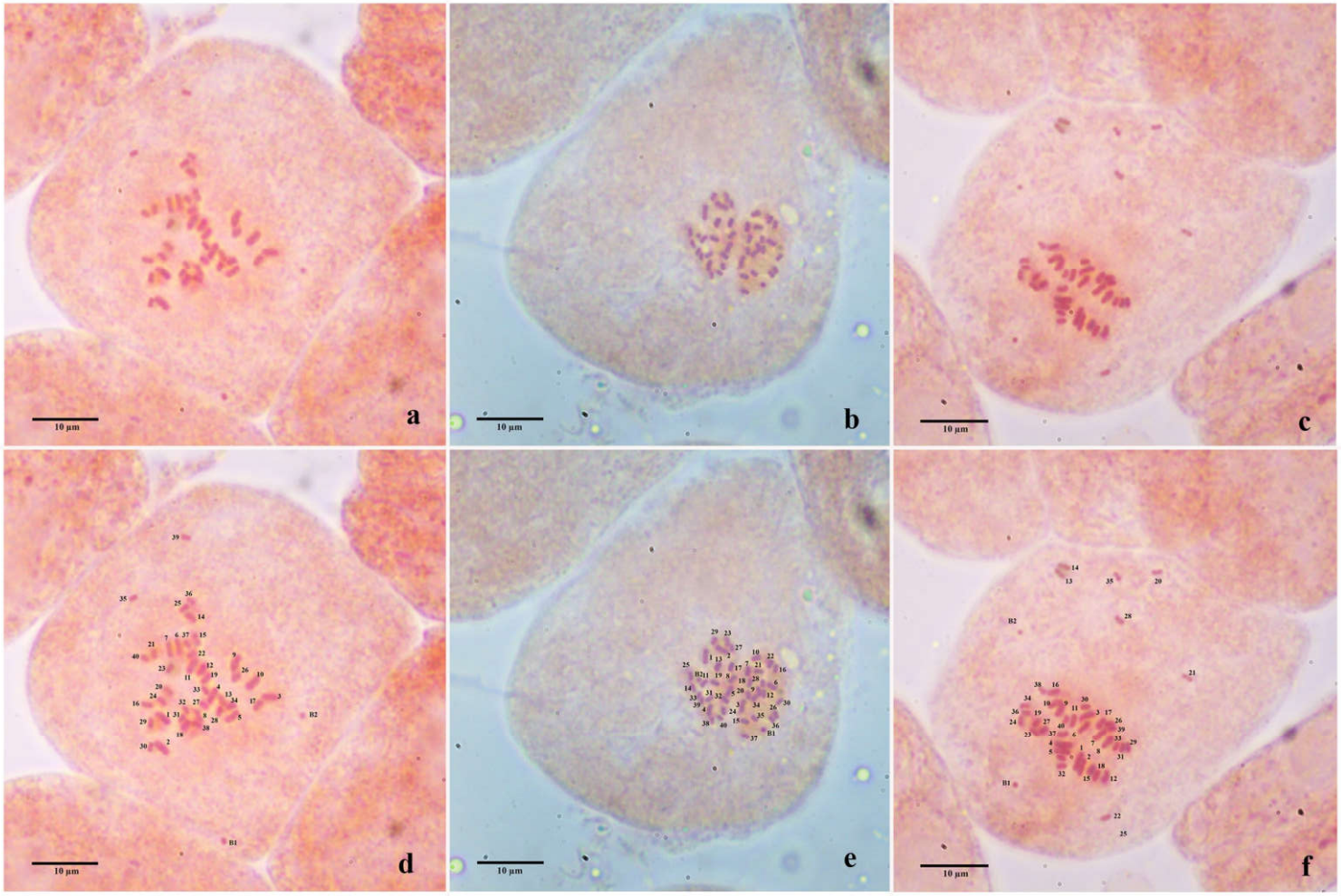


Plate 19: Somatic mitotic metaphase plates of *Strobilanthes virendrakumarana* with a variant chromosome number of 40+0-2B, a-c. Cells at mitotic metaphase; d-f. Karyotype with 40 chromosomes and 0-2 B chromosomes

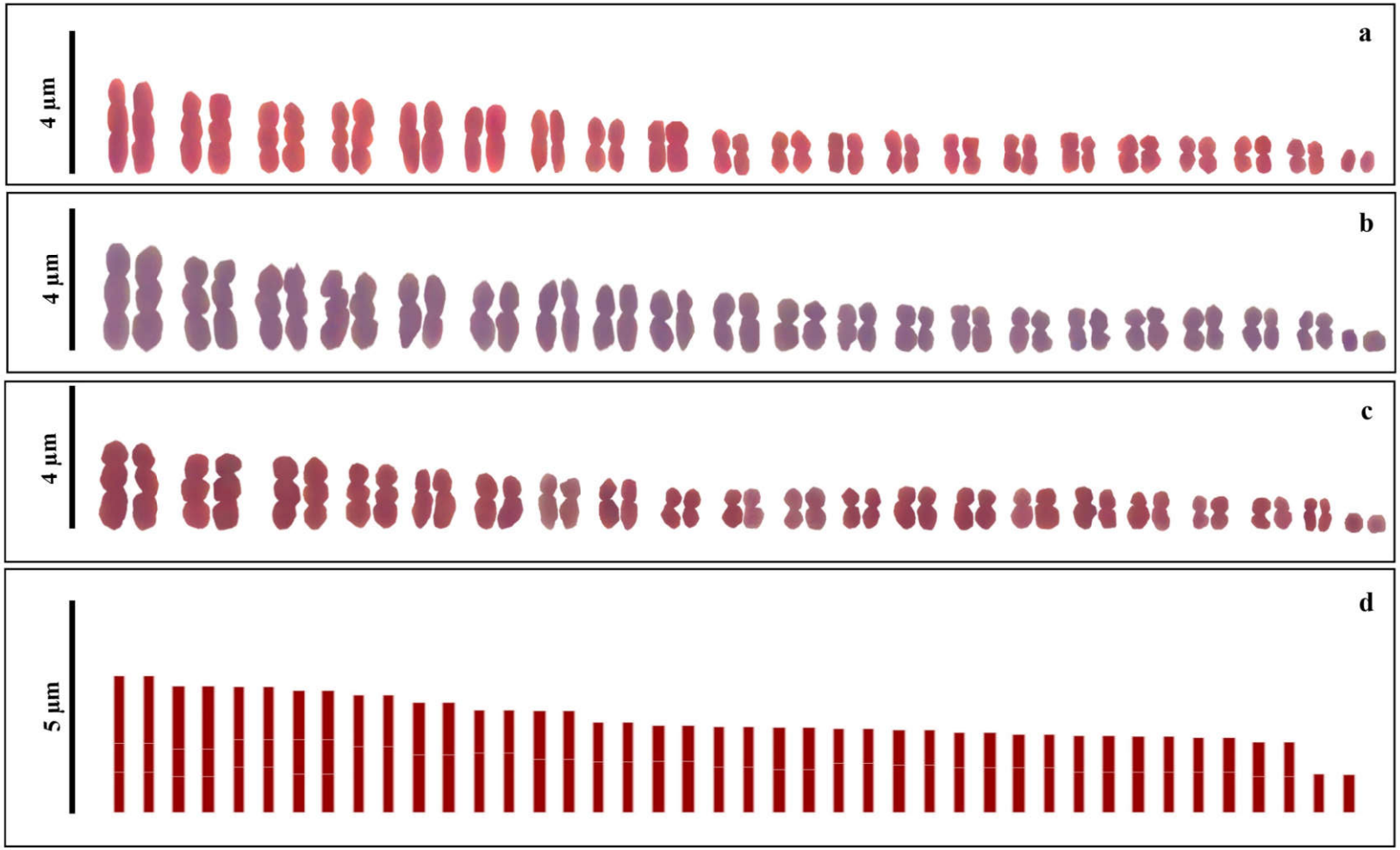


Plate 20: Mitotic variant chromosome complement images of *Strobilanthes virendrakumarana* with 40 chromosomes and 0-2B chromosomes, a-c. Karyograms; d. Idiogram

cytotypes 10 + 0-1B, 30 + 0-2B and 40 + 0-2B were 2 nsm (-) + 8 nm + 0-1 fragment, 30 nm + 0-2 fragments and 6 M + 34 nm + 0-2 fragments respectively. Within the species the chromosome length ranges from 1.34 to 2.95 µm (**Table 16**). The Rec index was high in the variant cytotype with 10 chromosomes but the As K% was high in 2n = 20. The TF% was lowest in 2n = 20 with 43.5%. The various asymmetric indices were also calculated.

The detailed karyomorphometric data of different ploidy levels were calculated and the parameters to assess karyotype symmetry or asymmetry and evolution status were summarized. It is based on the difference in chromosome length and the centromere position. For the cytological identity of the plant, karyograms of all the ploidy levels were drawn.

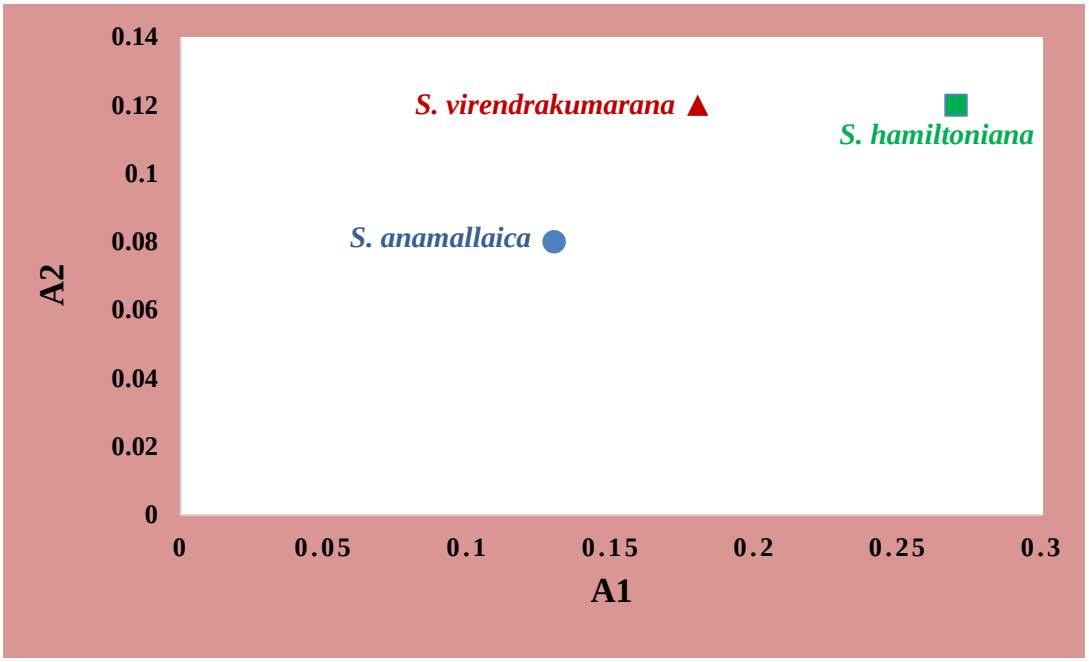


Figure 3: Scatter plot of intrachromosomal (A1) versus interchromosomal (A2) asymmetry indices of the taxa of *Strobilanthes*

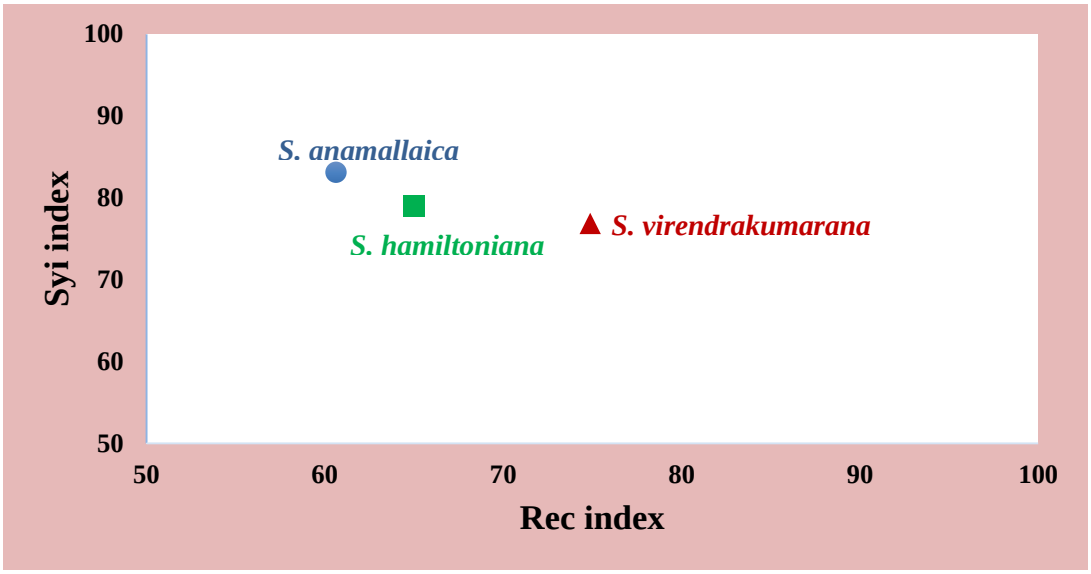


Figure 4: Scatter plot of Rec index (index of chromosome size resemblance) versus Syi index (index of karyotype symmetry) of the taxa of *Strobilanthes*

1. PHYTOCHEMICAL CHARACTERISATION

PHASE II – PHYTO-PHARMACOGNOSTIC PROFILING

Phytochemical characterization

The major phytochemical classes in plants include terpenes, phenols, alkaloids, saponins, phlobatannins, coumarins *etc*. These constituents synergistically act with each other to enhance the bioactivities and pharmacological importance of a plant. A single analytical technique is worthless to find out the potential phytocompounds in a plant. The techniques *viz.*, GC/MS, LC/MS, HR-LC/MS, and LC/NMR are some of them to identify the potential constituents.

a) Qualitative phytochemical estimation

The chemical synthesis of secondary metabolites of plants is not economically feasible. Hence these can be produced only by culturing plants which produces abundant secondary metabolites. Due to the high requirement of alkaloids, they are commercially prepared by cell and tissue culture, chemical semi-synthesis and biotechnological approaches.

Table 17: Preliminary phytochemical screening of selected species of *Strobilanthes*

Sl. No.	Phytochemical class of components	Tests	*S. anamallaica*			*S. hamiltoniana*			*S. virendrakumarana*		
			DW	Met	Hex	DW	Met	Hex	DW	Met	Hex
1.	Alkaloids	Wagner's test	-	+	-	-	+	-	+	+	-
		Hager's test	-	+	+	-	+	+	+	+	+
2.	Flavonoids	Alkaline reagent test	+	+	-	+	+	-	+	+	-
		Lead acetate test	+	+	-	-	+	-	+	+	-
3.	Phenols	Ferric chloride test	+	+	-	-	+	-	+	+	-
4.	Tannins	Breymer's test	-	-	-	-	-	-	-	-	-
		Ferric chloride test	-	-	-	-	-	-	-	-	-
5.	Terpenoids	Salkowski's test	+	+	-	+	+	-	+	+	+
6.	Saponins	Foam test	-	-	-	-	-	-	-	-	-
7.	Glycosides	Keller Killiani test	+	+	+	-	+	-	+	+	+
8.	Phlobatannins	HCl test	-	-	-	-	-	-	-	-	-
9.	Coumarins	Alcoholic NaOH test	+	-	-	+	-	-	+	-	-
10.	Resin	Turbidity test	-	-	-	-	-	-	-	-	-

(**SA**: *S. anamallaica*, **SH**: *S. hamiltoniana,* **SV**: *S. virendrakumarana*; +: presence; -: absence, DW – distilled water extract, Met – methanolic extract, Hex – hexane extract)

The presence and absence of various phytochemicals in the different crude extracts were analysed qualitatively. It was based on the colour formation and precipitation reactions. For this, three solvents were selected. They were distilled water, methanol and hexane. The phytochemical groups of chemicals in all the three extracts were summarised (**Table 17**). Alkaloids, flavonoids, phenols, terpenoids and glycosides were present in all the three species in which the first four class of compounds is in appreciable amount. The total absence of saponin, tannin, resin and phlobatannin was observed in the present study. Coumarins were present in aqueous extracts only. The number of compounds and amount of precipitation were high in methanolic extracts of all species. Therefore, further study was done in methanolic extracts.

b) Quantitative phytochemical estimation

The quantitative estimation of the major class of compounds revealed the exact content of them within the extract. The quantitative estimation of total phenol, total flavonoid, total alkaloid and total terpenoids were analysed. In total phenolic content, *S. hamiltoniana* (88.88 ± 5.55 mg GAE/g DW) recorded the highest value against gallic acid (**Figure 5**). It is followed by *S. virendrakumarana* and *S. anamallaica* (**Figure 6**). The content was almost double in two species when compared with *S. hamiltoniana* phenol content. The flavonoid content was tested against quercetin (**Figure 7**). It was high in *S. virendrakumarana* with a value of 124.82 ± 1.3 mg QE/g DW. The flavonoid content was very low in *S. hamiltoniana* (**Figure 8**). Almost high content in all the three species were recorded during alkaloid estimation (**Figure 10**). It was high in *S. virendrakumarana* (50.2 ± 2.96 mg CE/g DW) against caffeine (**Figure 9**). Despite of all the other three estimations, terpenoid content was recorded very low in *S. hamiltoniana,* it was only 6.75 ± 0.72 mg against linalool (**Figure 11**). But the terpenoid content was almost equal in *S. anamallaica* and *S. virendrakumarana* (**Figure 12**).

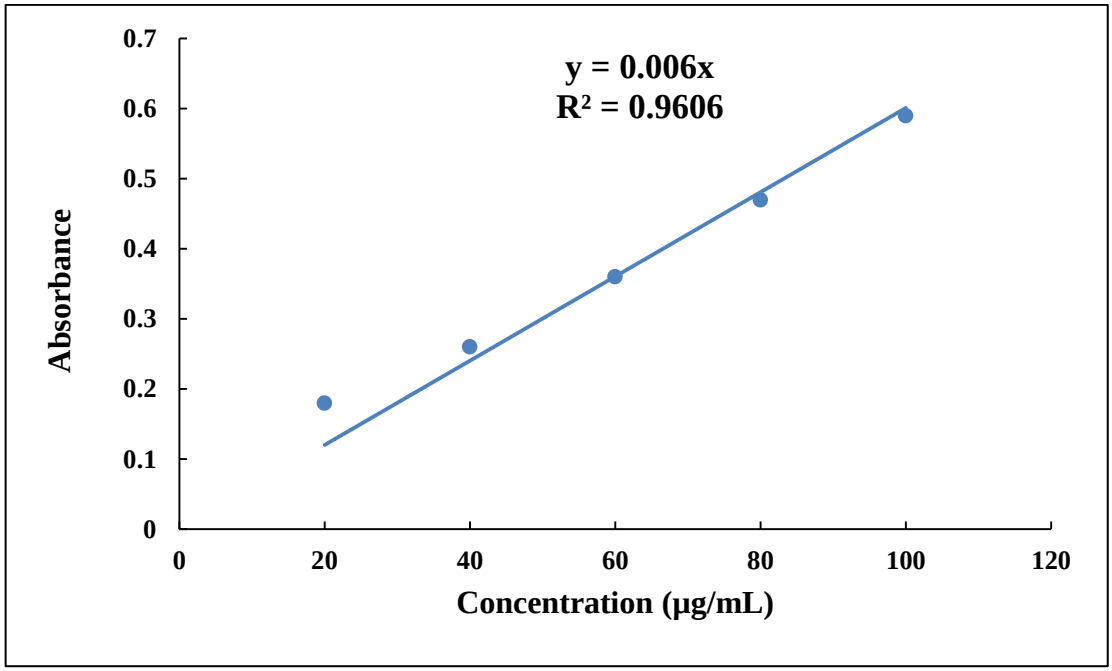


Figure 5: Standard calibration curve of gallic acid for the determination of total phenolic content

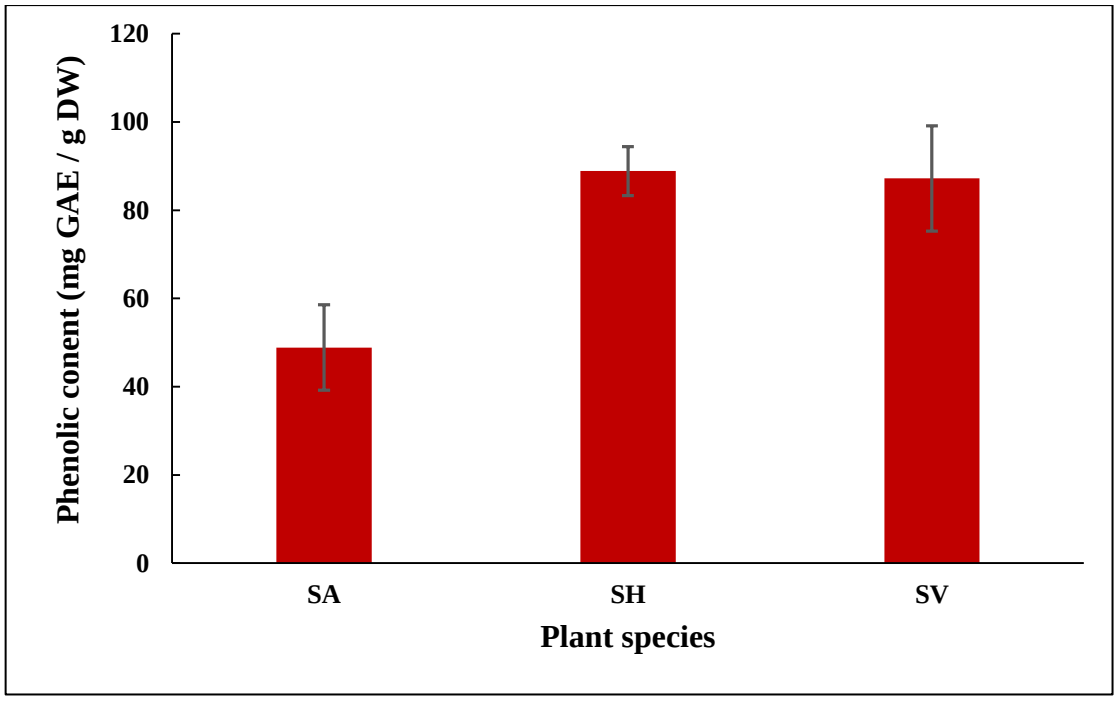


Figure 6: Quantitative estimation of total phenolic content of selected species of *Strobilanthes*: SA - *Strobilanthes anamallaica*; SH - *Strobilanthes hamiltoniana*; SV - *Strobilanthes virendrakumarana*.

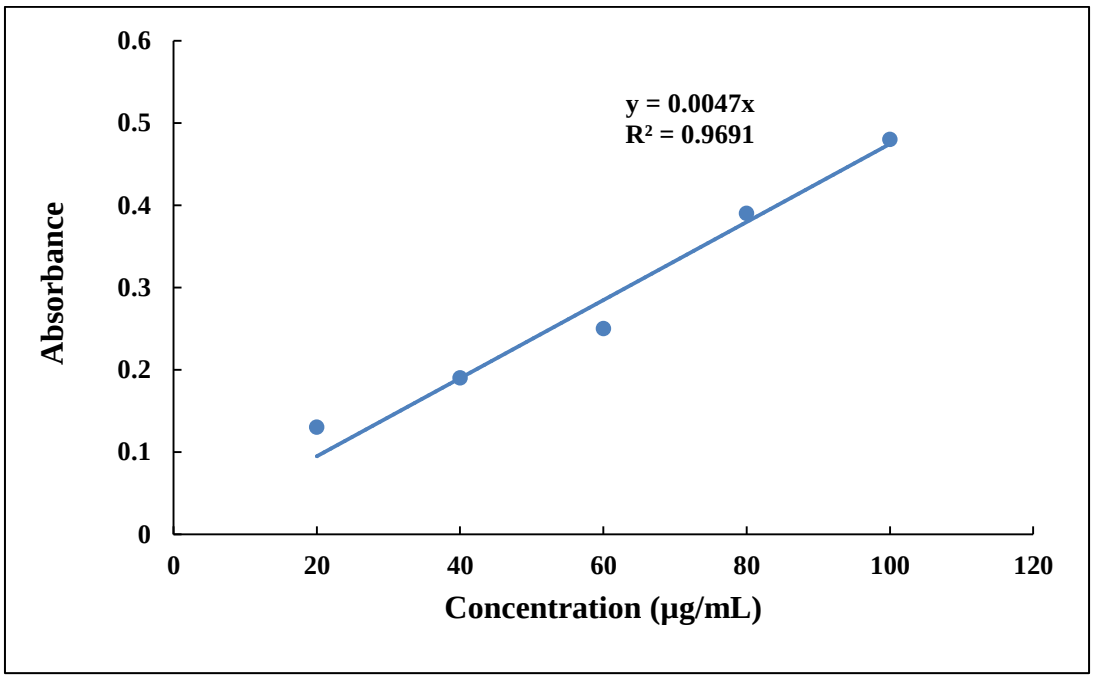


Figure 7: Standard calibration curve of quercetin for the determination of total flavonoid content

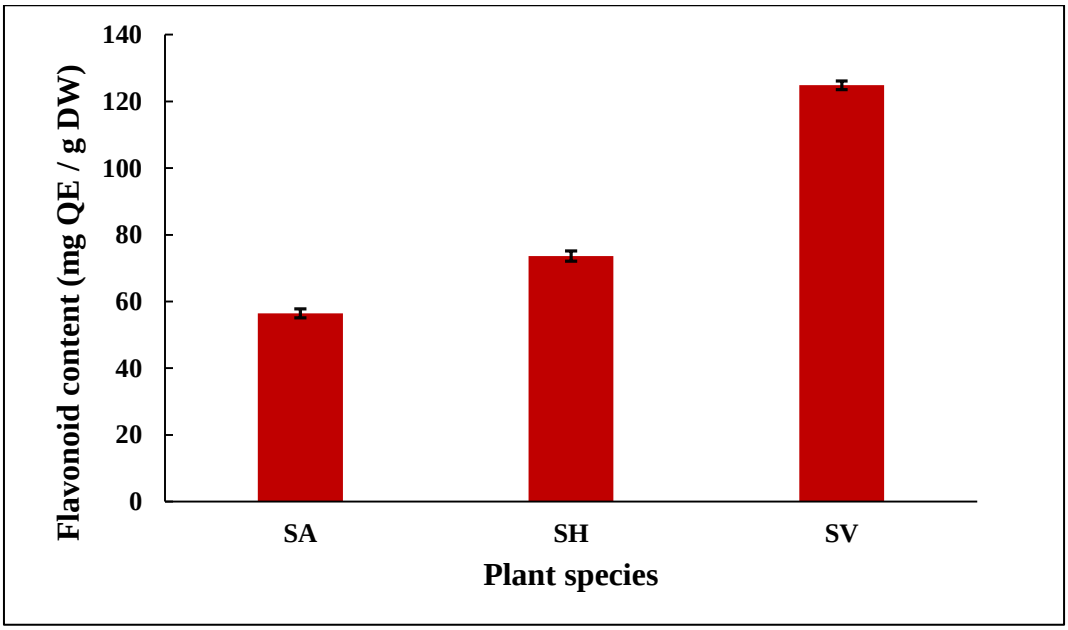


Figure 8: Quantitative estimation of total flavonoid content of selected species of *Strobilanthes*: SA - *Strobilanthes anamallaica*; SH - *Strobilanthes hamiltoniana*; SV - *Strobilanthes virendrakumarana*.

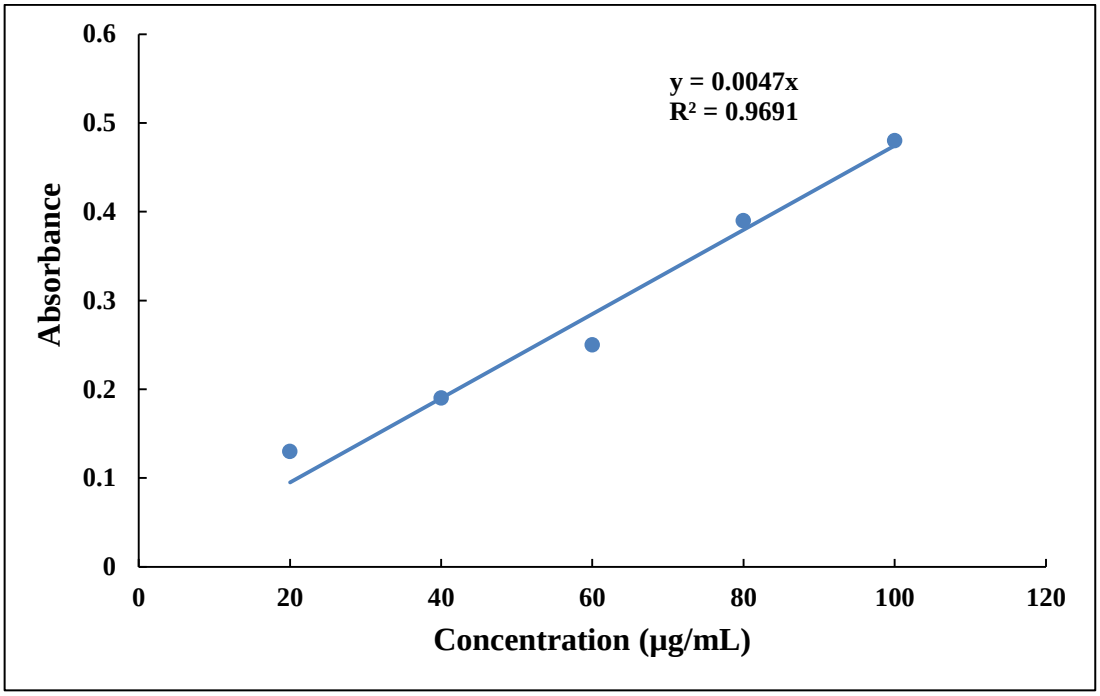


Figure 9: Standard calibration curve of caffeine for the determination of total alkaloid content

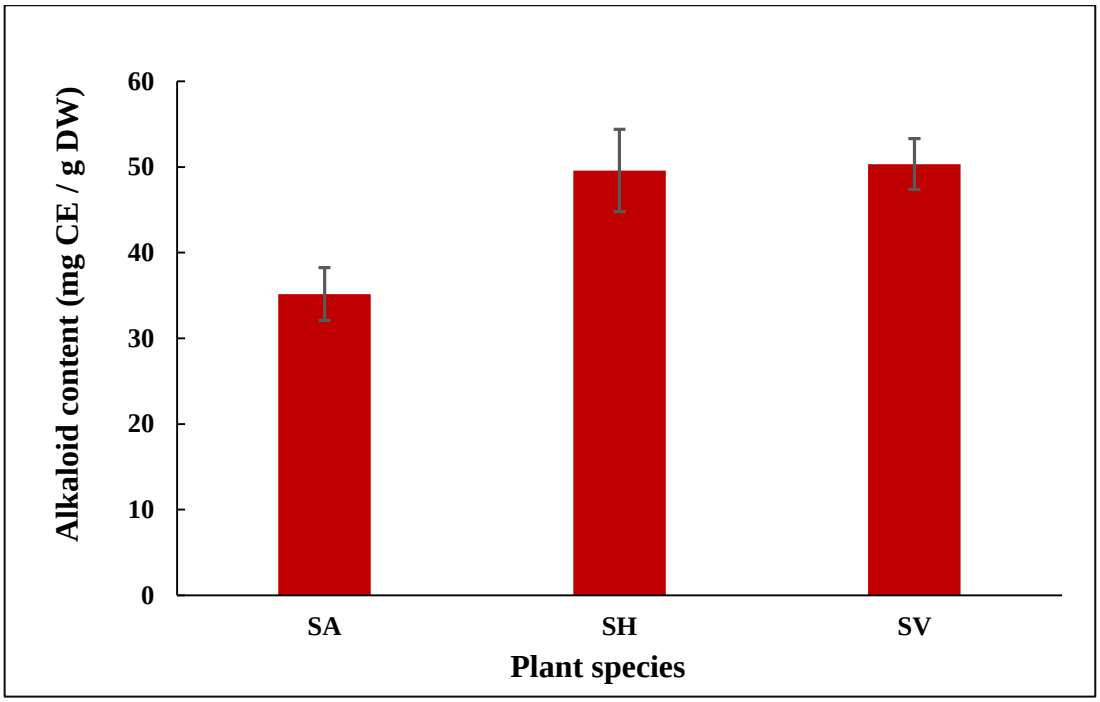


Figure 10: Quantitative estimation of total alkaloid content of selected species of *Strobilanthes*: SA - *Strobilanthes anamallaica*; SH - *Strobilanthes hamiltoniana*; SV - *Strobilanthes virendrakumarana*.

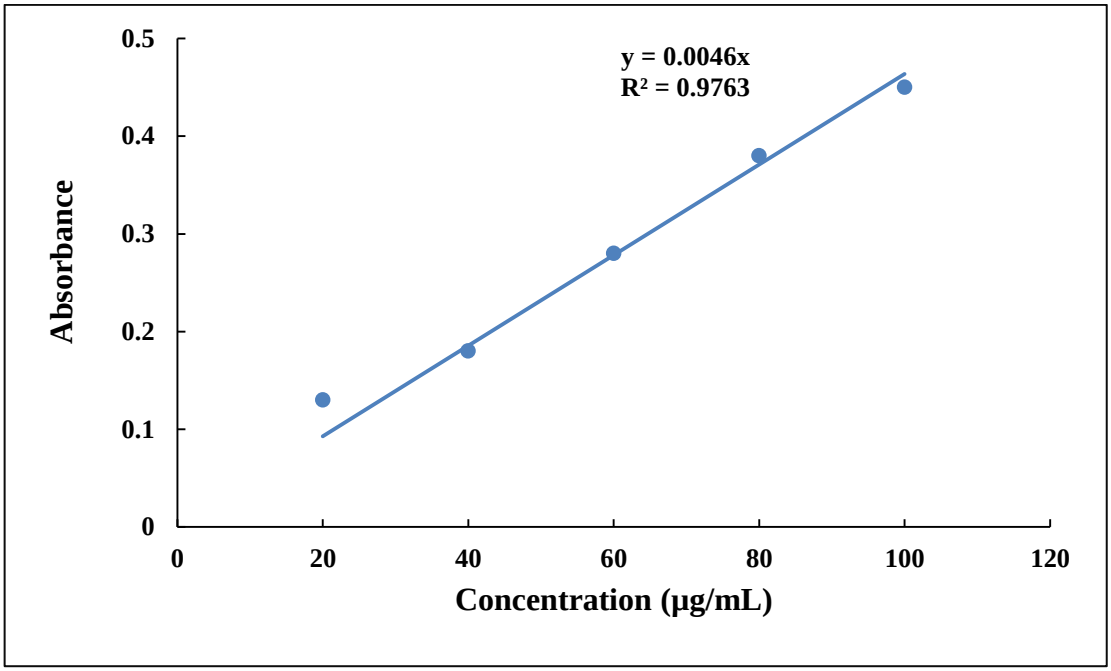


Figure 11: Standard calibration curve of linalool for the determination of total terpenoid content

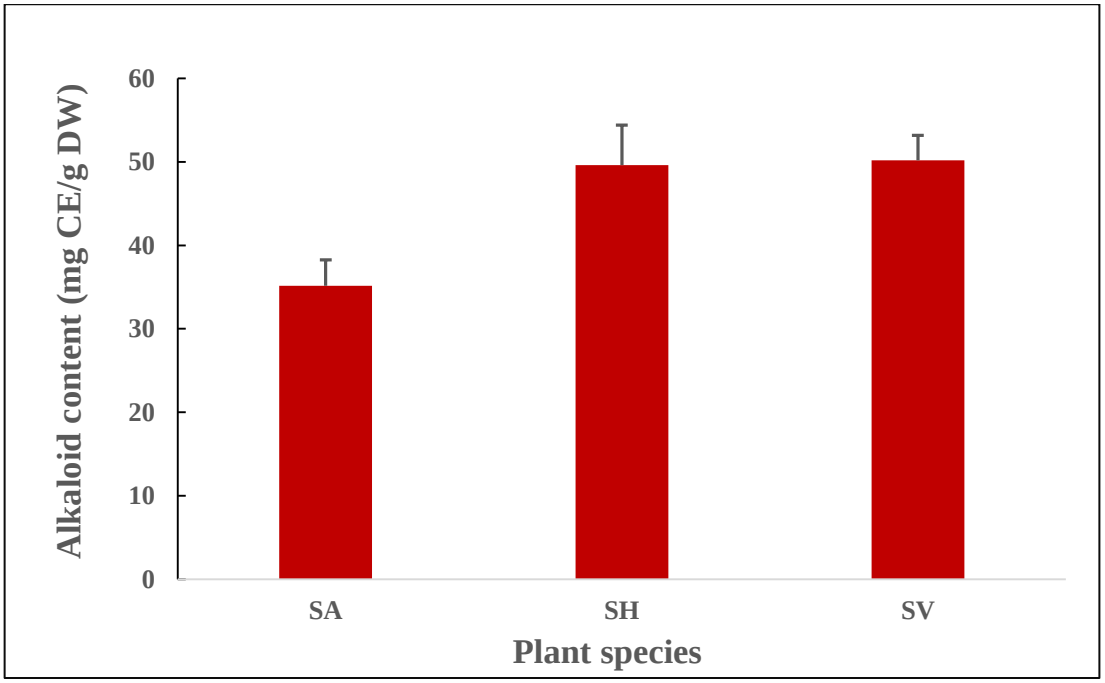


Figure 12: Quantitative estimation of total terpenoid content of selected species of *Strobilanthes*: SA - *Strobilanthes anamallaica*; SH - *Strobilanthes hamiltoniana*; SV - *Strobilanthes virendrakumarana*.

c) Gas chromatography/Mass Spectrometry (GC/MS) analysis

The GC/MS analytical method is a combination of gas chromatography and mass spectrometry, to reveal the volatile phytoconstituents in an extract with its area percentage. The components are resolved based on their retention time. The identification was confirmed on the basis of peak area, retention time and molecular formula (**Figures 13, 14, 15**). The GC/MS analysis of the shoot methanolic extracts of the three *Strobilanthes* species revealed a total of 49 phytocompounds (**Table 18**). They were resolved within the range of 6.601 to 47.621 retention time. The mass spectra of individual compounds are shown in **Figure 16 (i-xii)**. The first and the last compounds were heptanoic acid and thunbergol. Among the resolved ones, six compounds were found to be common in all the species with different area percentage. They were neophytadiene, palmitic acid, phytol, squalene, γ-sitosterol and lupeol. The intensity of the common compounds varies with species. Squalene was the compound with the highest content in *S. hamiltoniana.* Whereas α-linolenic acid and γ-sitosterol occupied the top position in *S. anamallaica* and *S. virendrakumarana* respectively.

Analysis for the phytochemicals from *S. anamallaica* opened up about 23 diverse constituents. The major among them were α-linolenic acid (32.19%), 5-hydroxymethylfurfural (20.68%), palmitic acid (9.34%) and lupeol (8.64%). The first and the last resolved constituent was 2-butoxypentane (RT = 6.735) and lupeol (RT = 40.674) respectively. About 15 compounds were solely present in *S. anamallaica.* The major ones were α-linolenic acid **and** 5-hydroxymethylfurfural.

S. hamiltoniana summed up a total of 19 compounds with a terpene called steviol is having the least peak area percentage. Apart from squalene (32.75%) other major compounds in the decreasing order of their peak area percentage were γ-sitosterol, phytol, lupeol, palmitic acid, stigmasta-5,22-

dien-3-ol and neophytadiene. The retention time of compounds ranges from 6.601 to 47.621. The first and the last resolved candidates were heptanoic acid and thunbergol repectively. Heptanoic acid, 2-hexenoic acid, dodecanoic acid, longiverbenone, decanoic acid, hexahydrofarnesyl acetone, methyl docosanoate, vitamin E, stigmasta-5,22-dien-3-ol and thunbergol were limited to *S. hamiltoniana.*

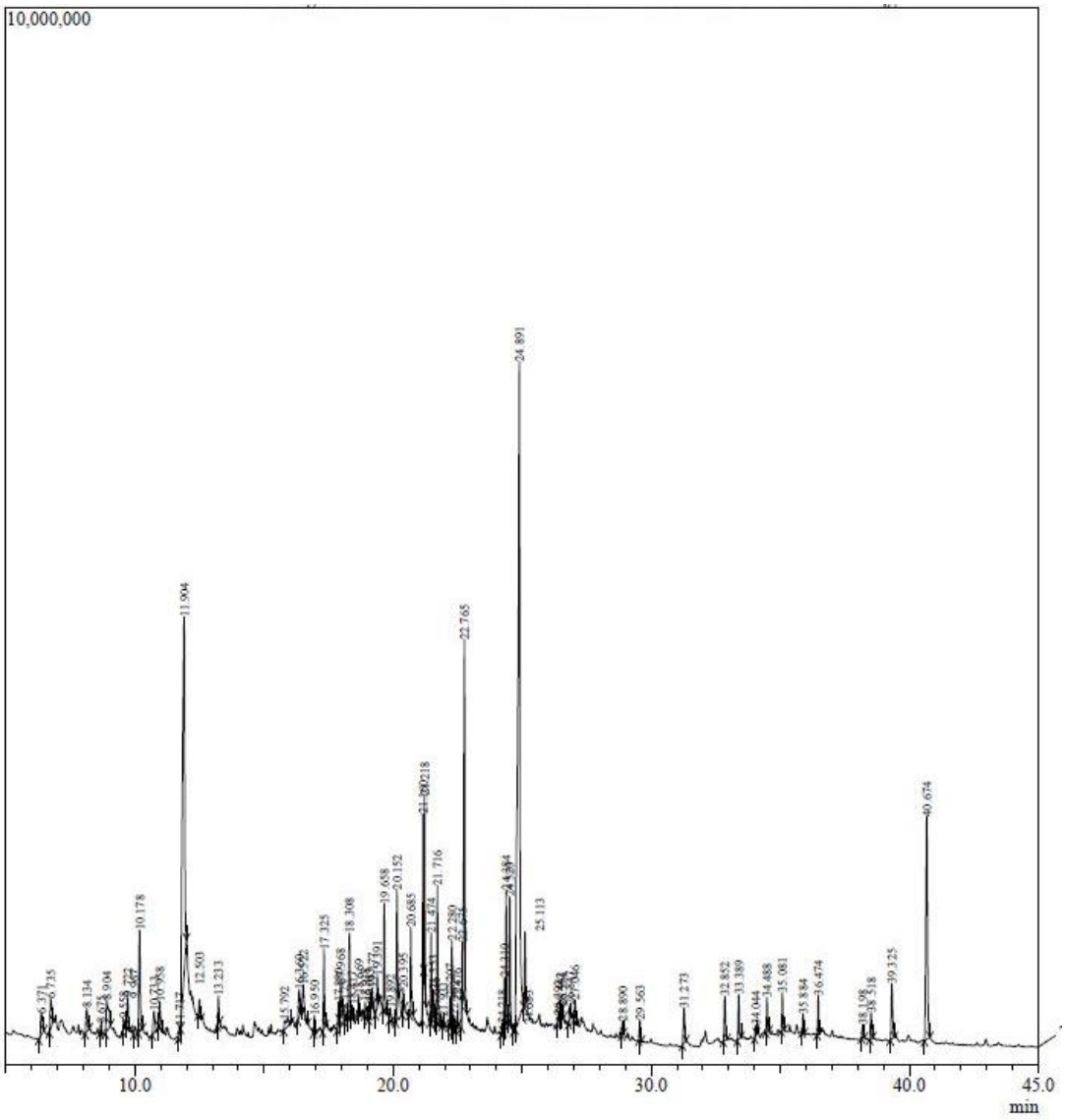


Figure 13: Gas chromatogram of methanolic extract of *S. anamallaica*

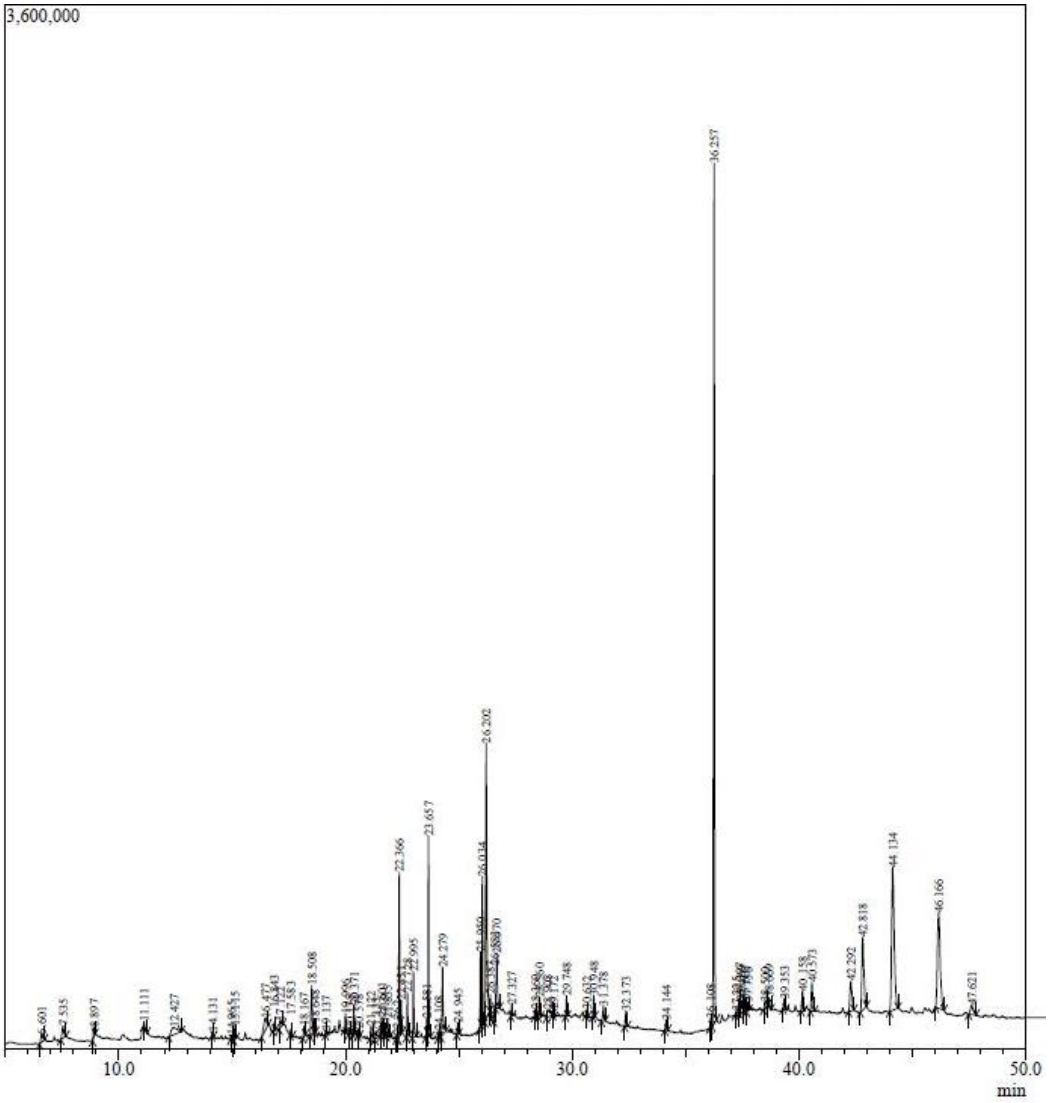


Figure 14: Gas chromatogram of methanolic extract of *S. hamiltoniana*

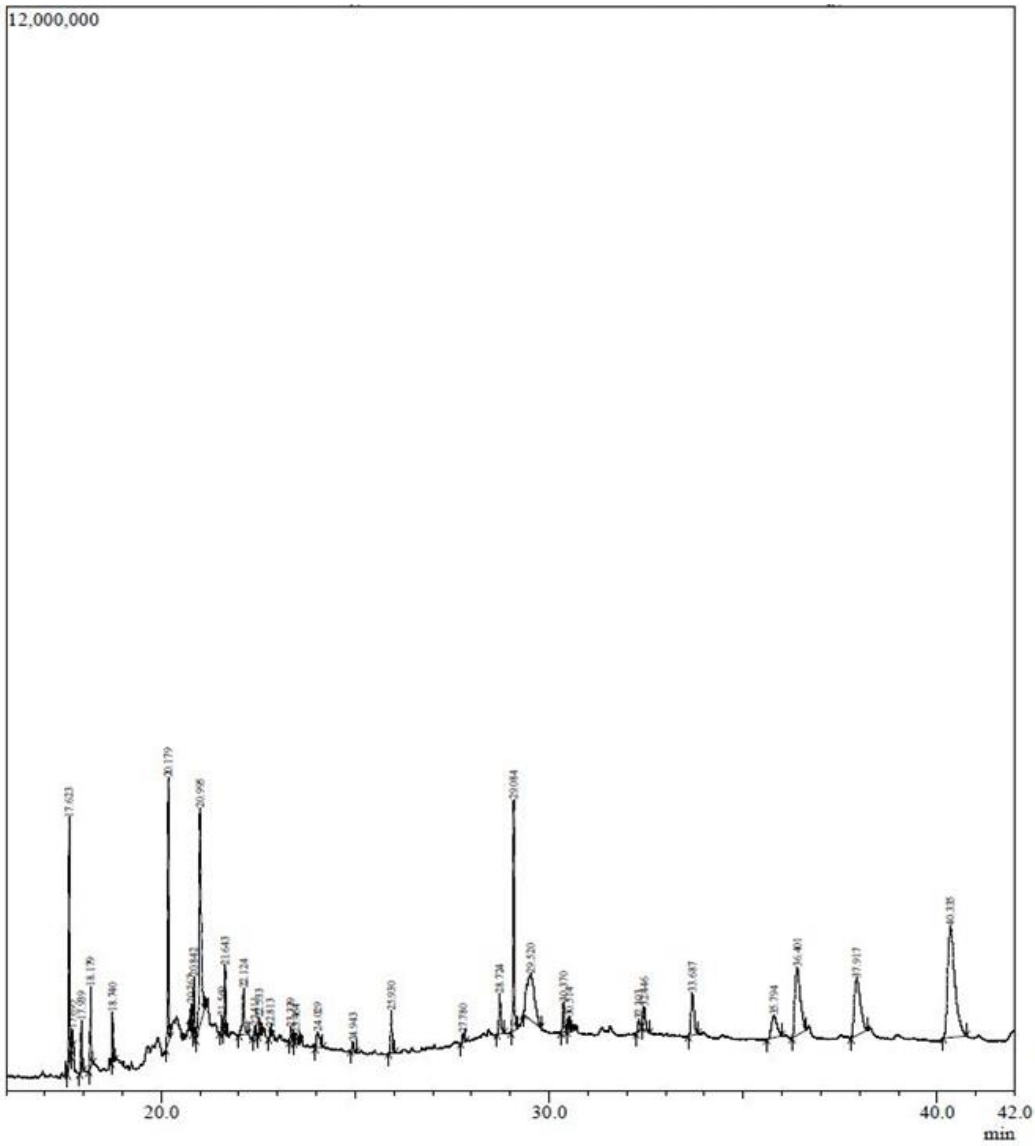


Figure 15: Gas chromatogram of methanolic extract of *S. virendrakumarana*

Within the 22 resolved compounds from *S. virendrakumarana*, γ-sitosterol (19.9%) was the highest intensity constituent. 3,6-Octadecadienoic acid, lupeol, campesterol, squalene and stigmasterol were the other major ones. The retention time ranged between 17.623 and 40.674. In the present study, 14 compounds were resolved in this species only. The major ones were methyl palmitate, 3,6-Octadecadienoic acid, Murolan-3,9(11)-diene-10-peroxy, campesterol and stigmasterol.

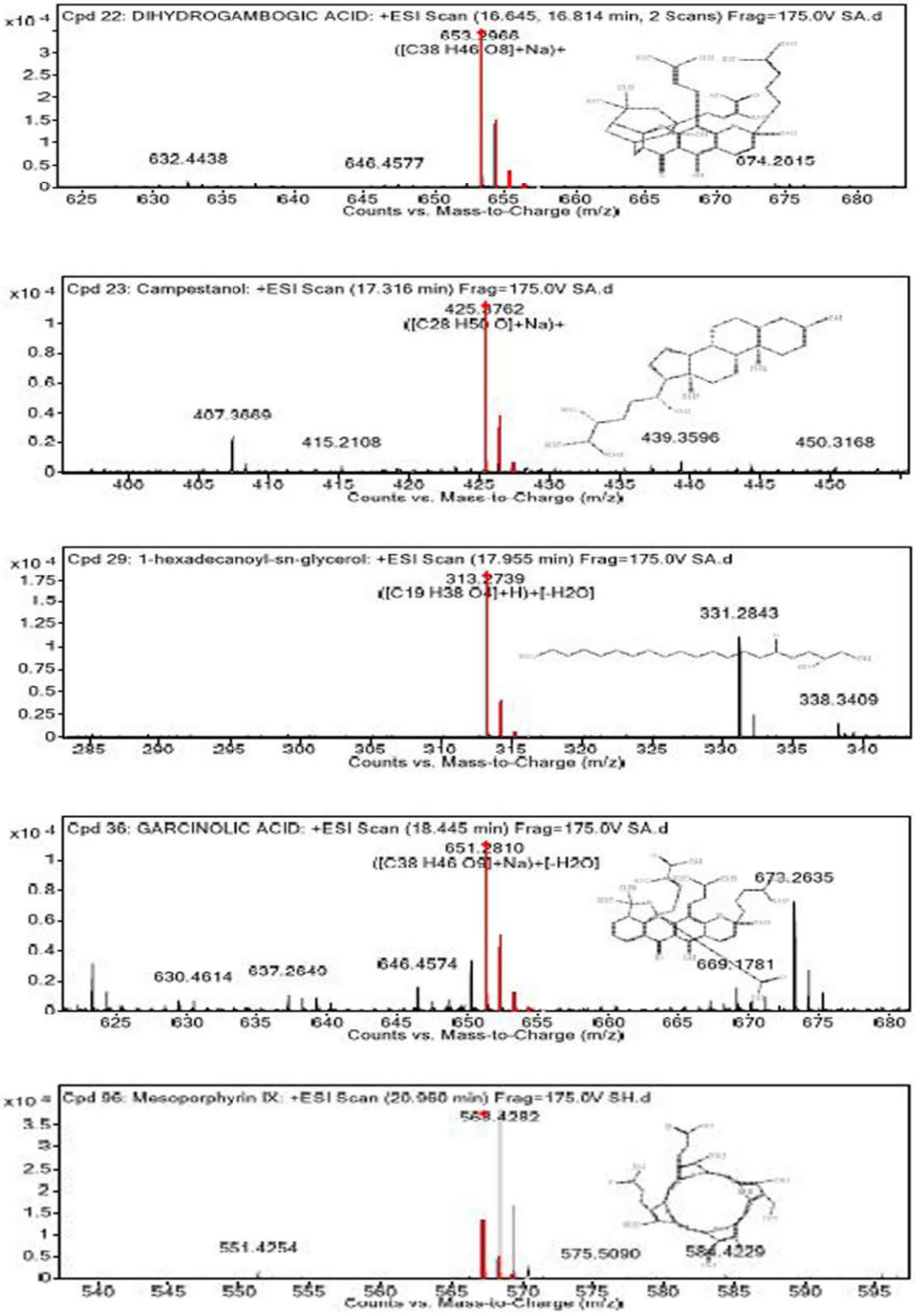


Figure 20 (v): Mass spectra of compounds detected by HR-LC/MS analysis of methanolic extract of selected species of *Strobilanthes*

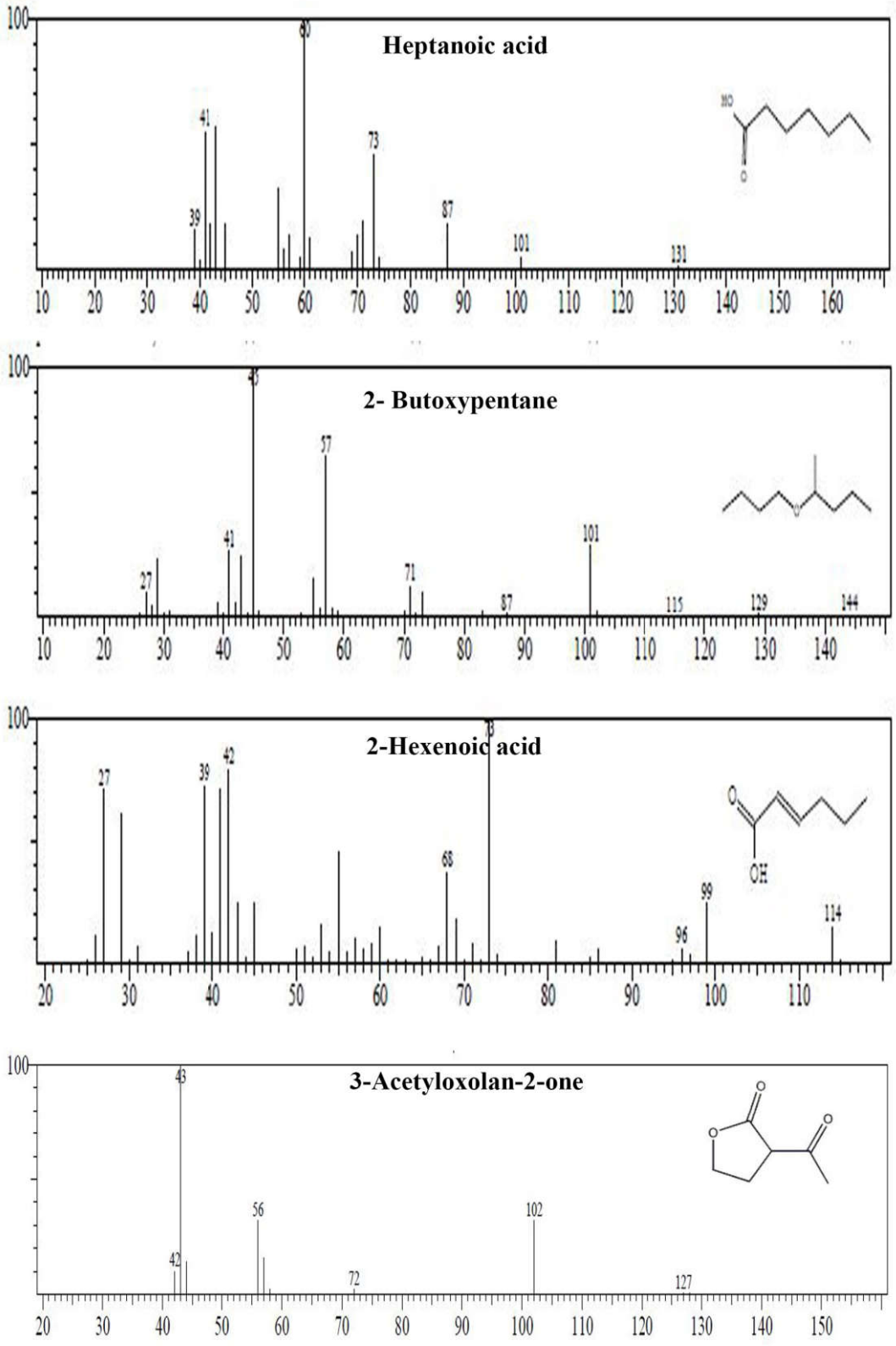


Figure 16 (i): Mass spectra of compounds detected by GC/MS analysis of methanolic extract of selected taxa of *Strobilanthes*

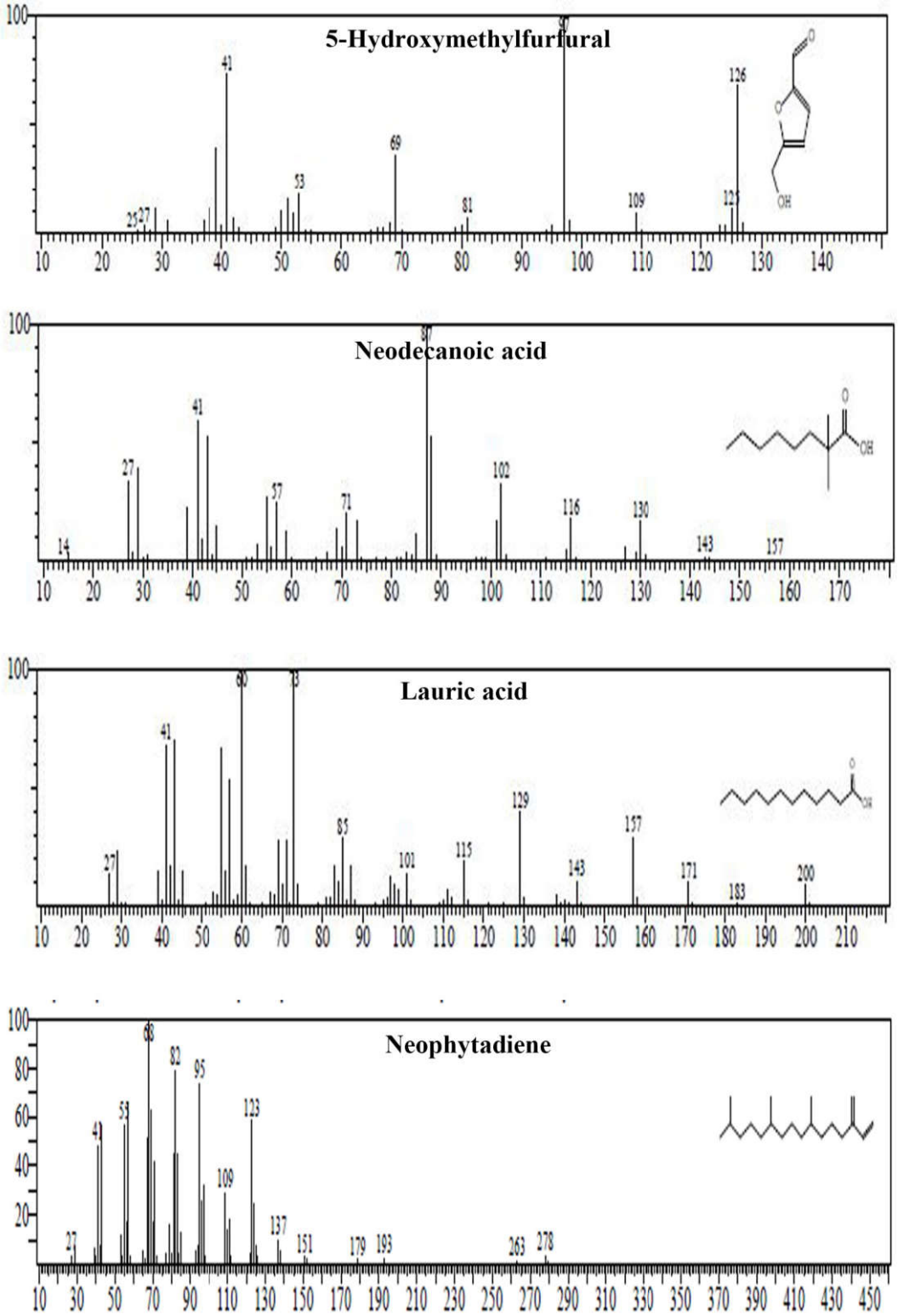


Figure 16 (ii): Mass spectra of compounds detected by GC/MS analysis of methanolic extract of selected taxa of *Strobilanthes*

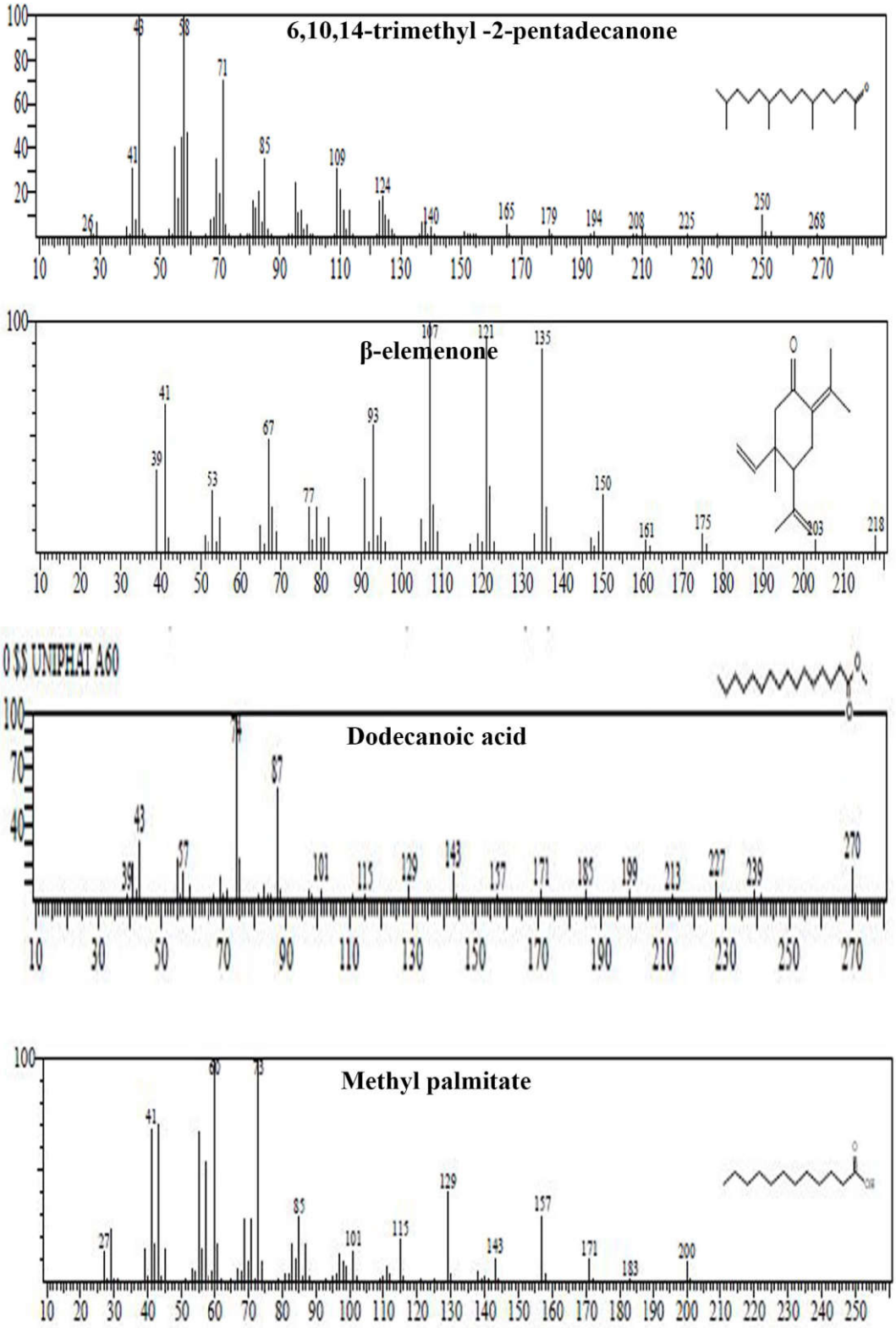


Figure 16 (iii): Mass spectra of compounds detected by GC/MS analysis of methanolic extract of selected taxa of *Strobilanthes*

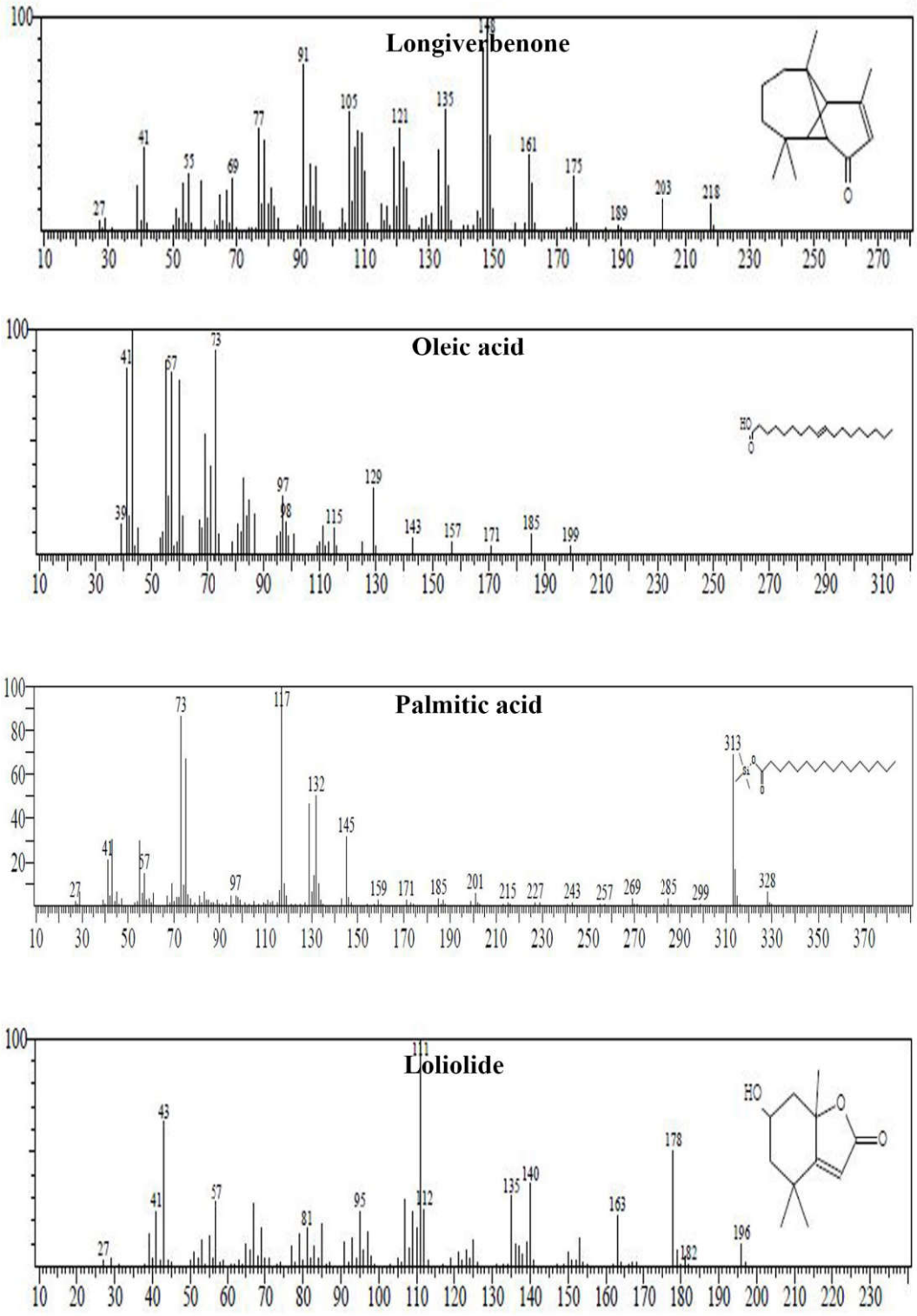


Figure 16 (iv): Mass spectra of compounds detected by GC/MS analysis of methanolic extract of selected taxa of *Strobilanthes*

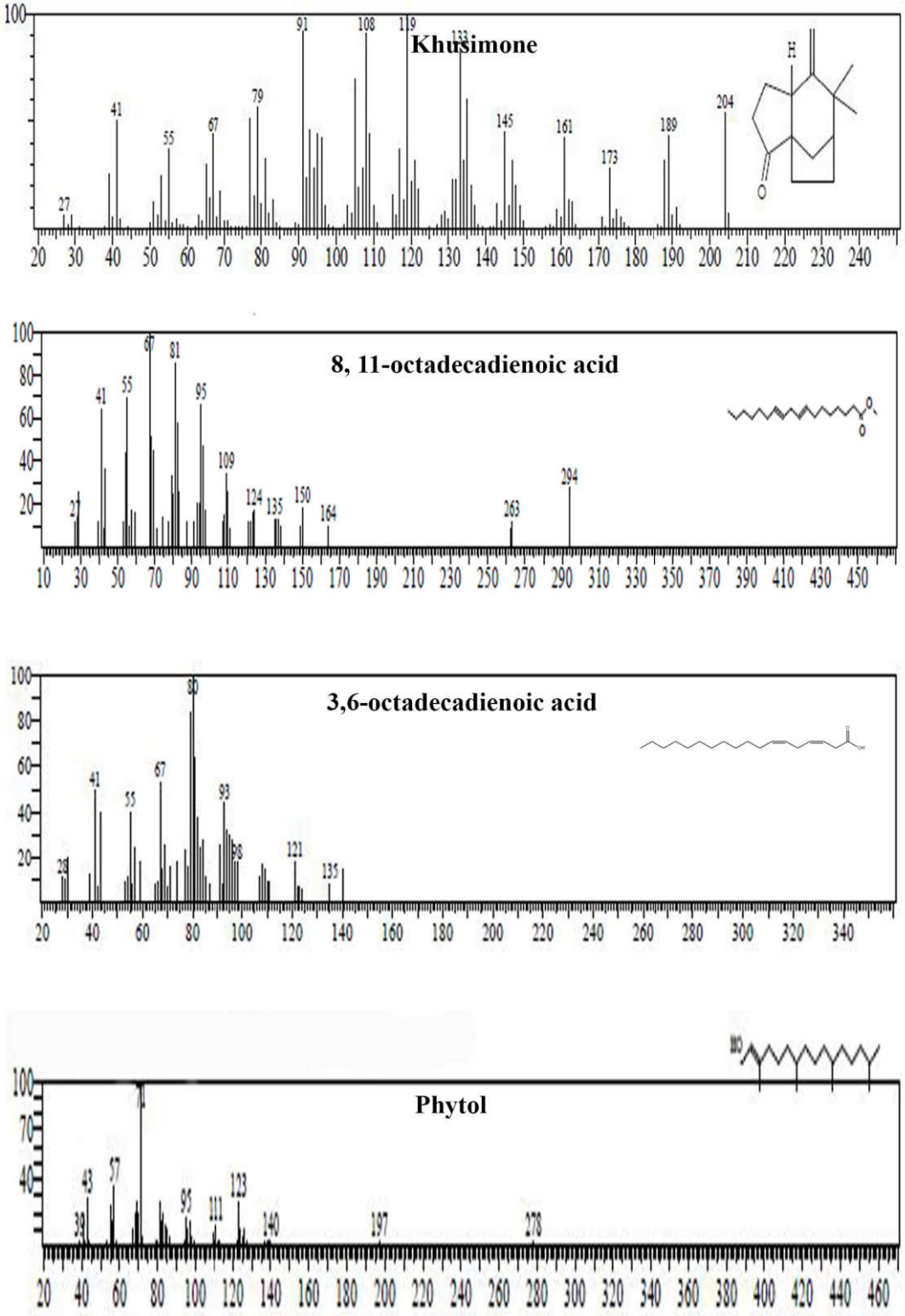


Figure 16 (v): Mass spectra of compounds detected by GC/MS analysis of methanolic extract of selected taxa of *Strobilanthes*

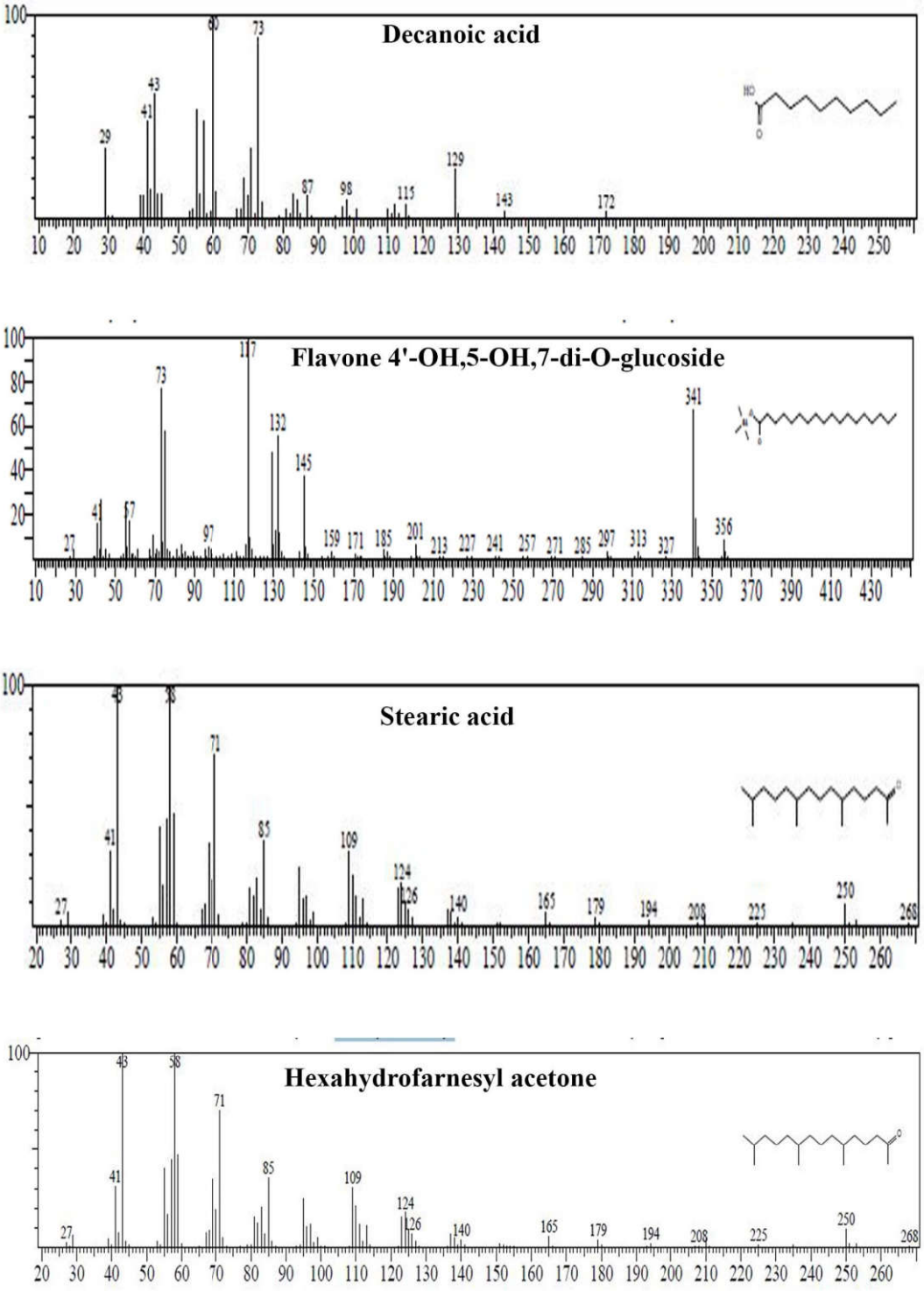


Figure 16 (vi): Mass spectra of compounds detected by GC/MS analysis of methanolic extract of selected taxa of *Strobilanthes*

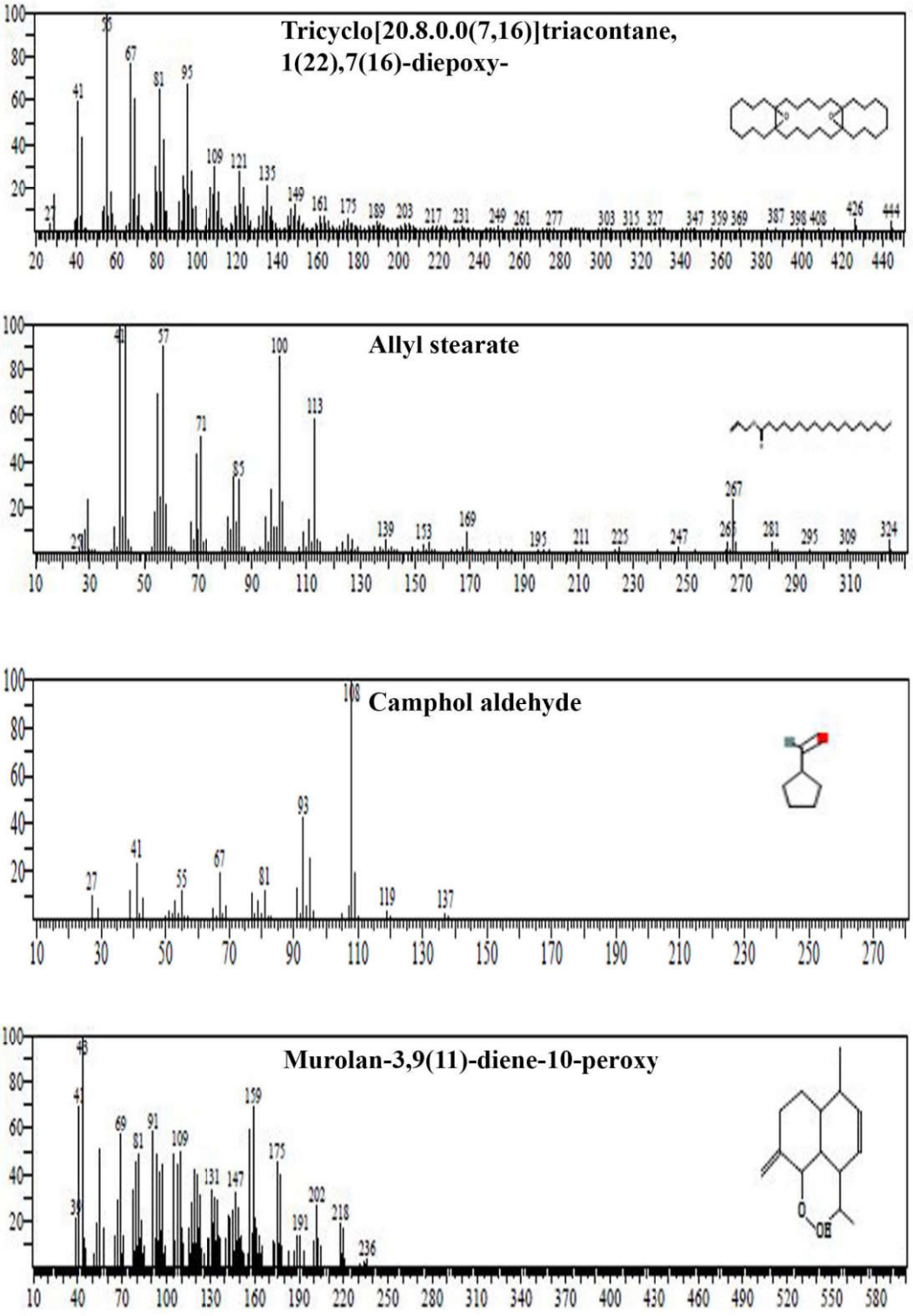


Figure 16 (vii): Mass spectra of compounds detected by GC/MS analysis of methanolic extract of selected taxa of *Strobilanthes*

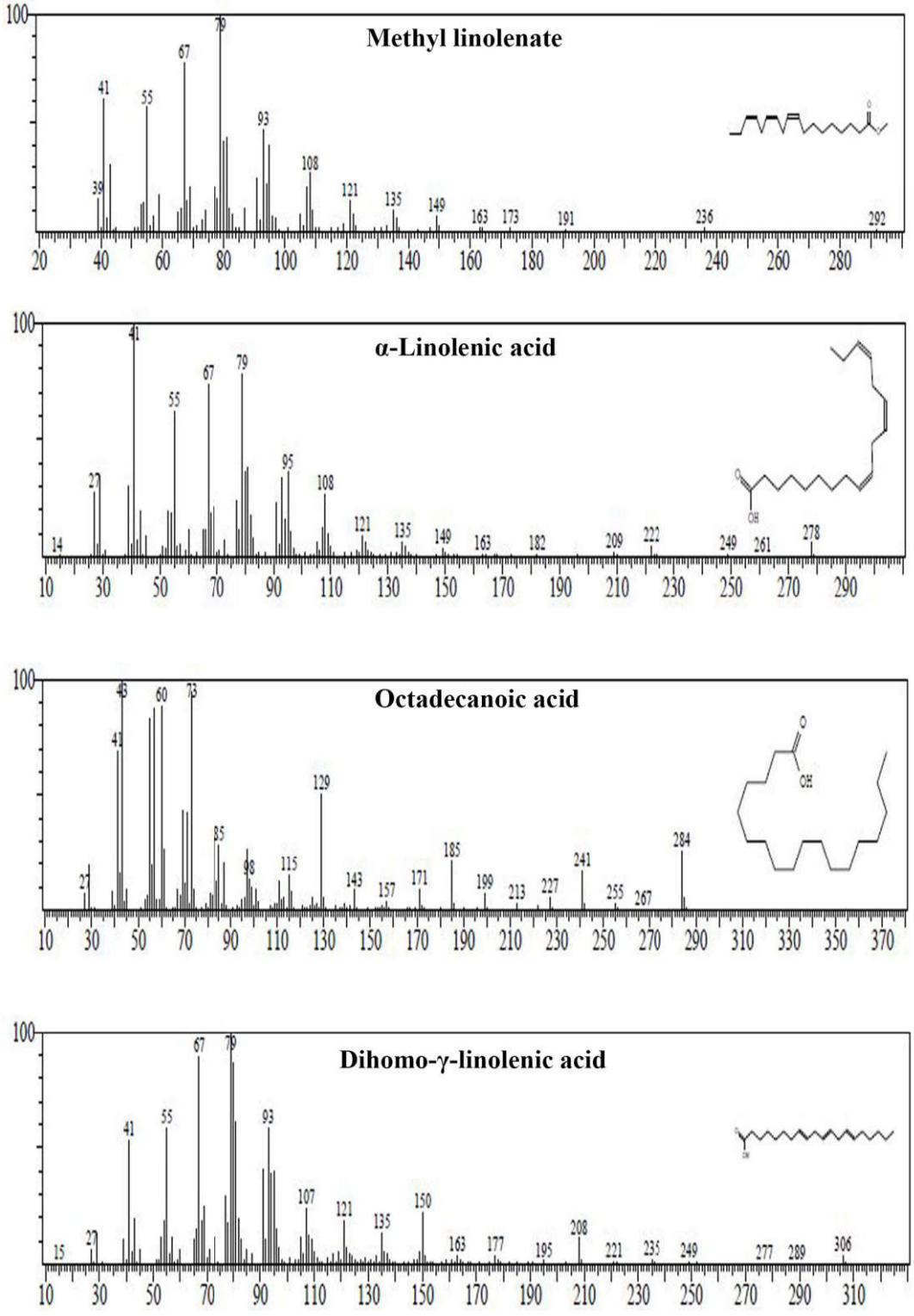


Figure 16 (viii): Mass spectra of compounds detected by GC/MS analysis of methanolic extract of selected taxa of *Strobilanthes*

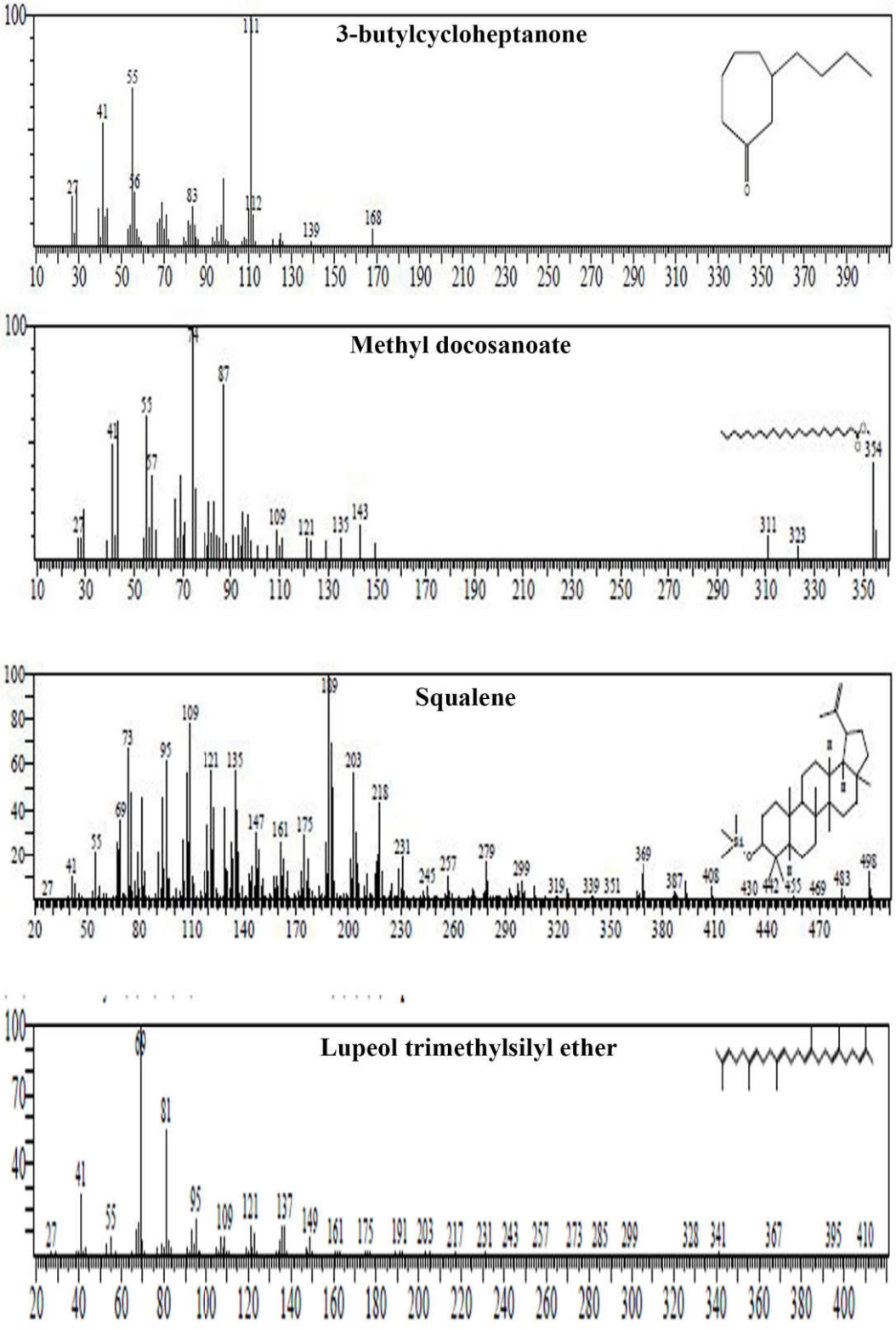


Figure 16 (ix): Mass spectra of compounds detected by GC/MS analysis of methanolic extract of selected taxa of *Strobilanthes*

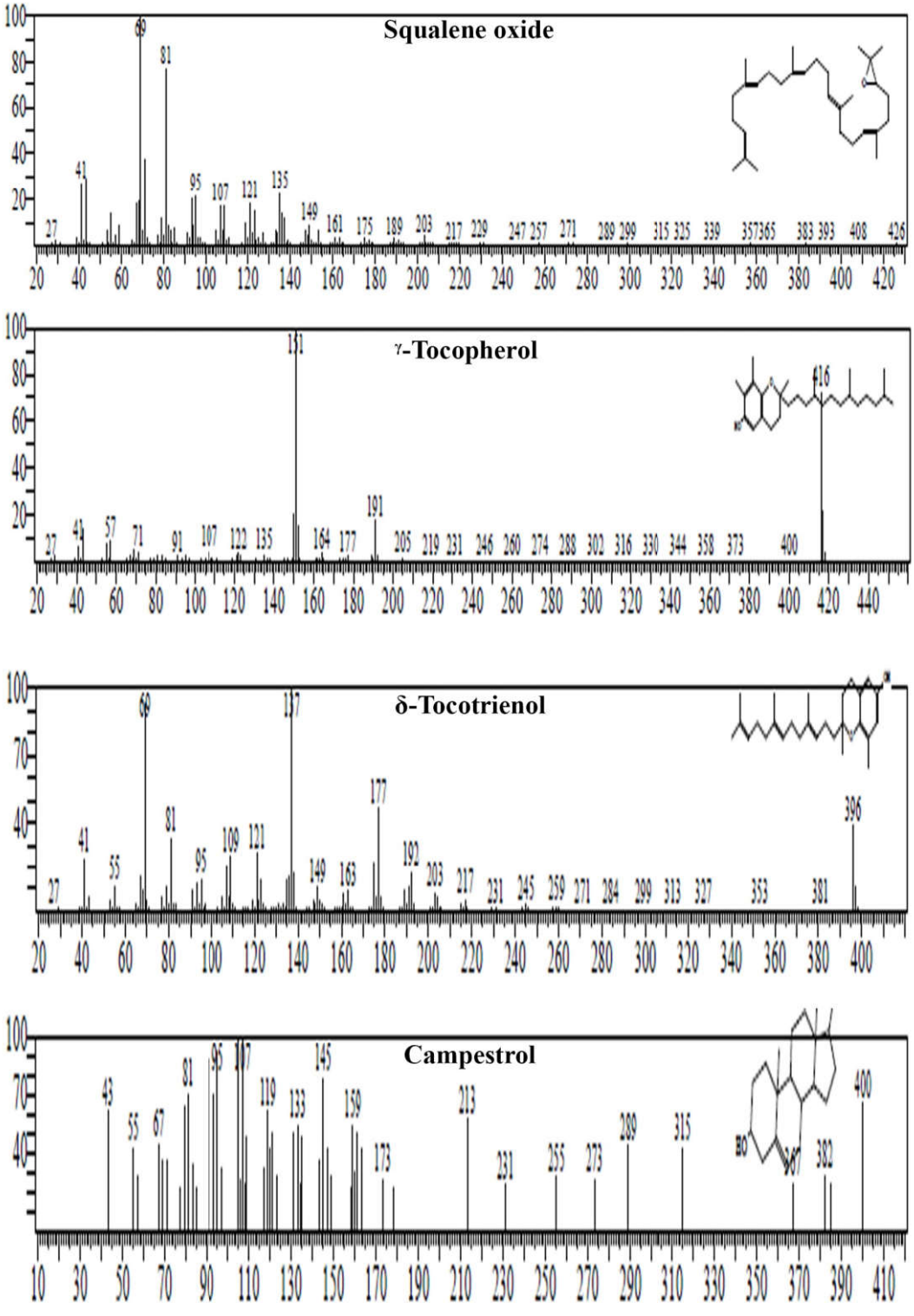


Figure 16 (x): Mass spectra of compounds detected by GC/MS analysis of methanolic extract of selected taxa of *Strobilanthes*

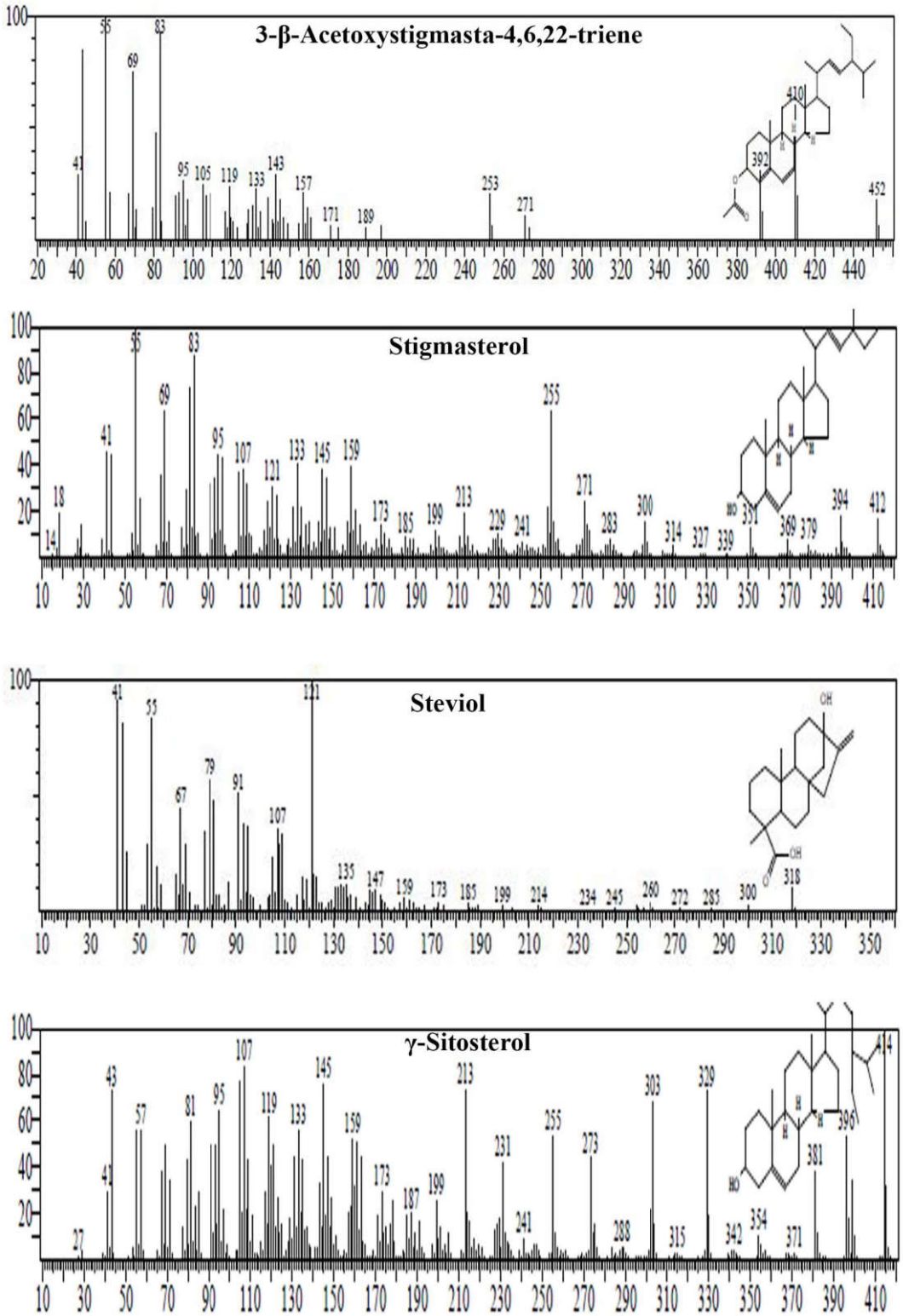


Figure 16 (xi): Mass spectra of compounds detected by GC/MS analysis of methanolic extract of selected taxa of *Strobilanthes*

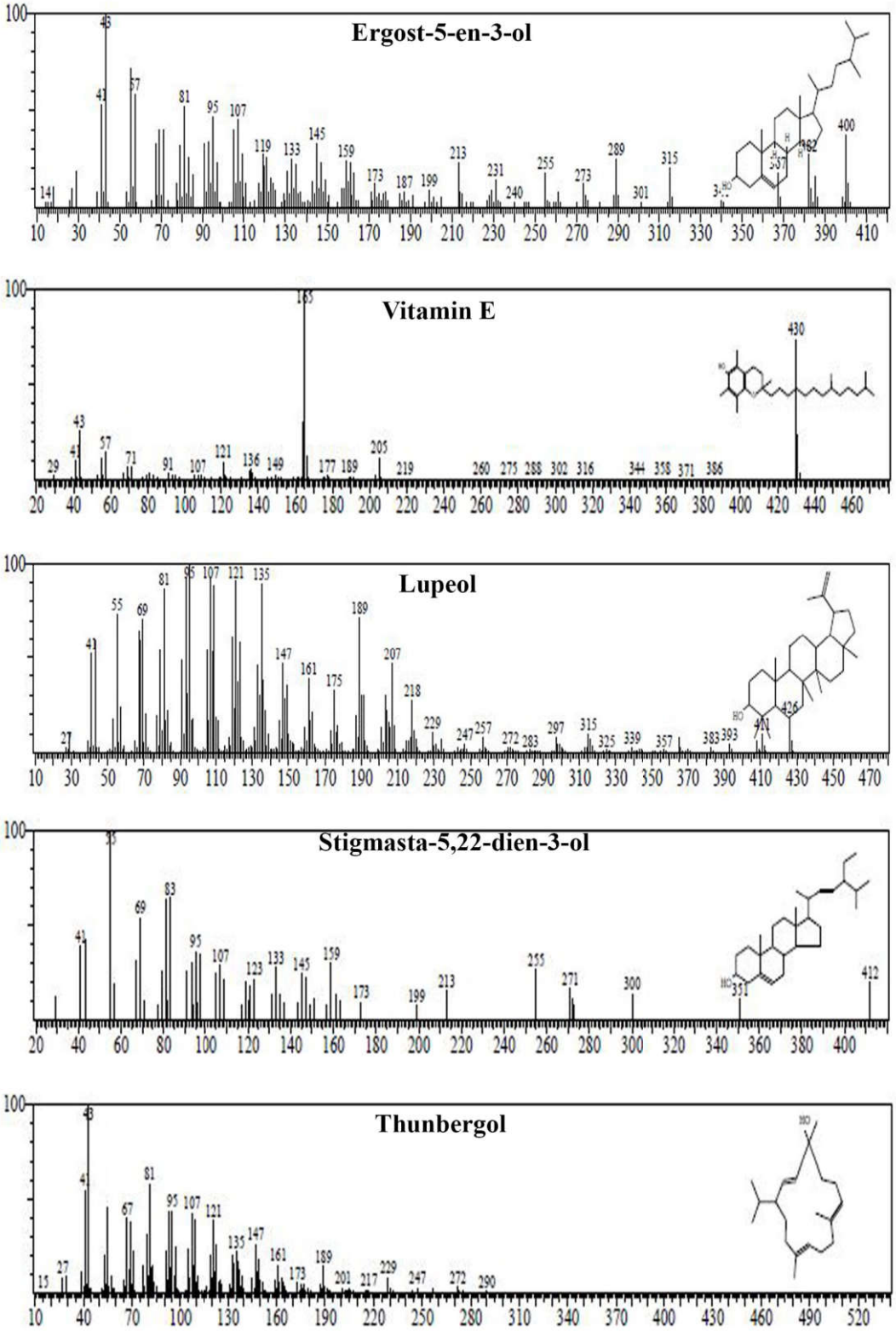


Figure 16 (xii): Mass spectra of compounds detected by GC/MS analysis of methanolic extract of selected taxa of *Strobilanthes*

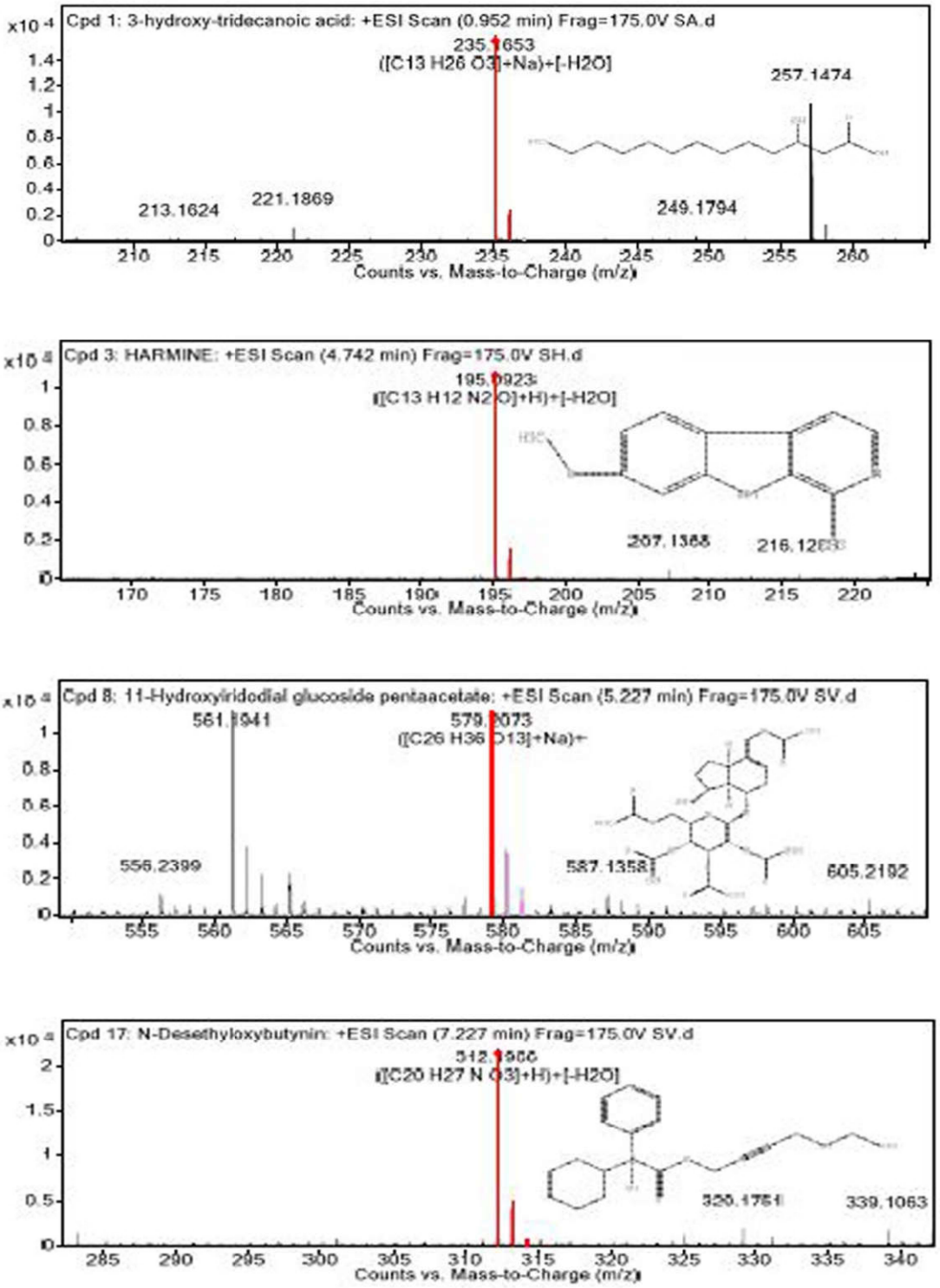


Figure 20 (i): Mass spectra of compounds detected by HR-LC/MS analysis of methanolic extract of selected species of *Strobilanthes*

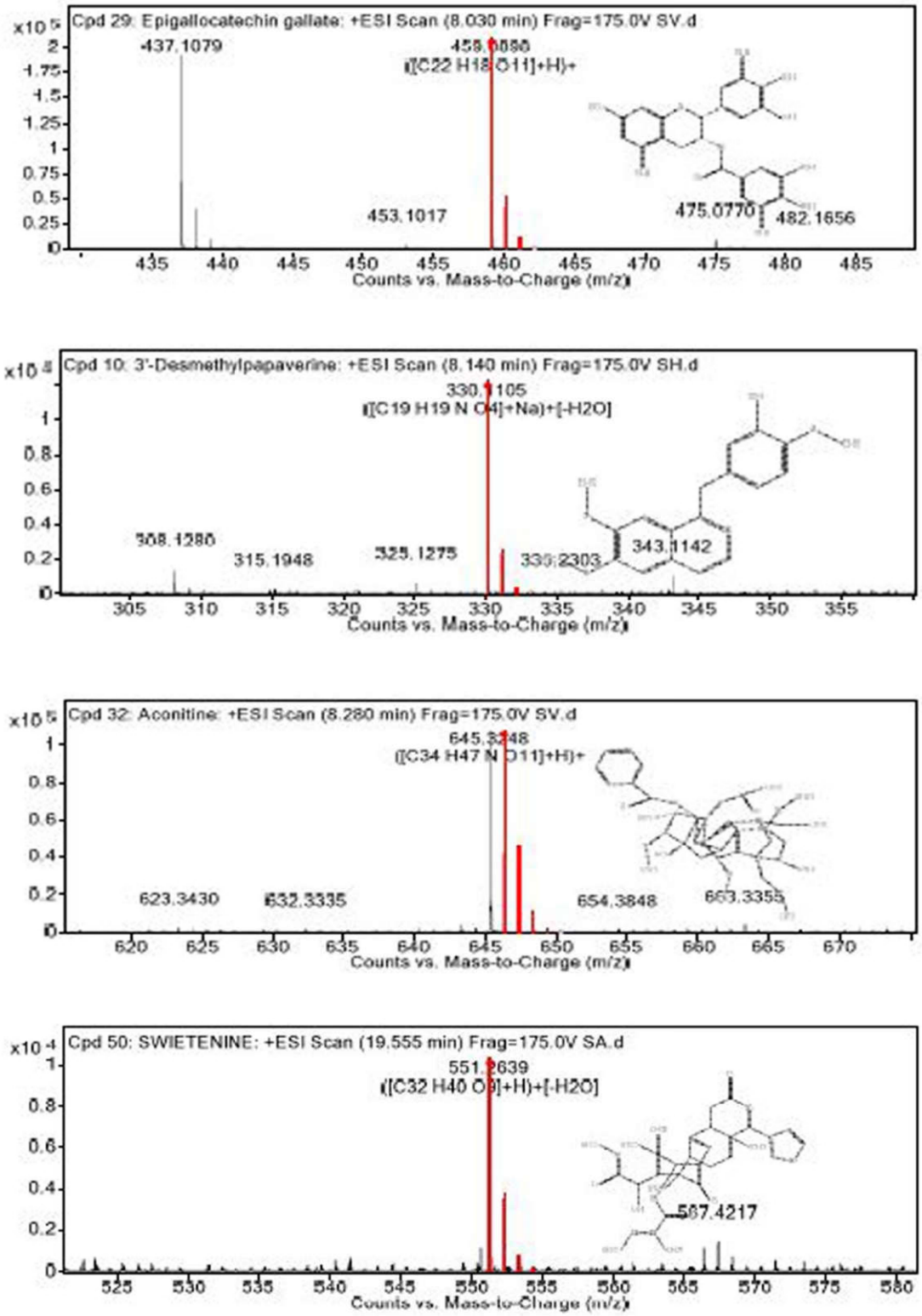


Figure 20 (ii): Mass spectra of compounds detected by HR-LC/MS analysis of methanolic extract of selected species of *Strobilanthes*

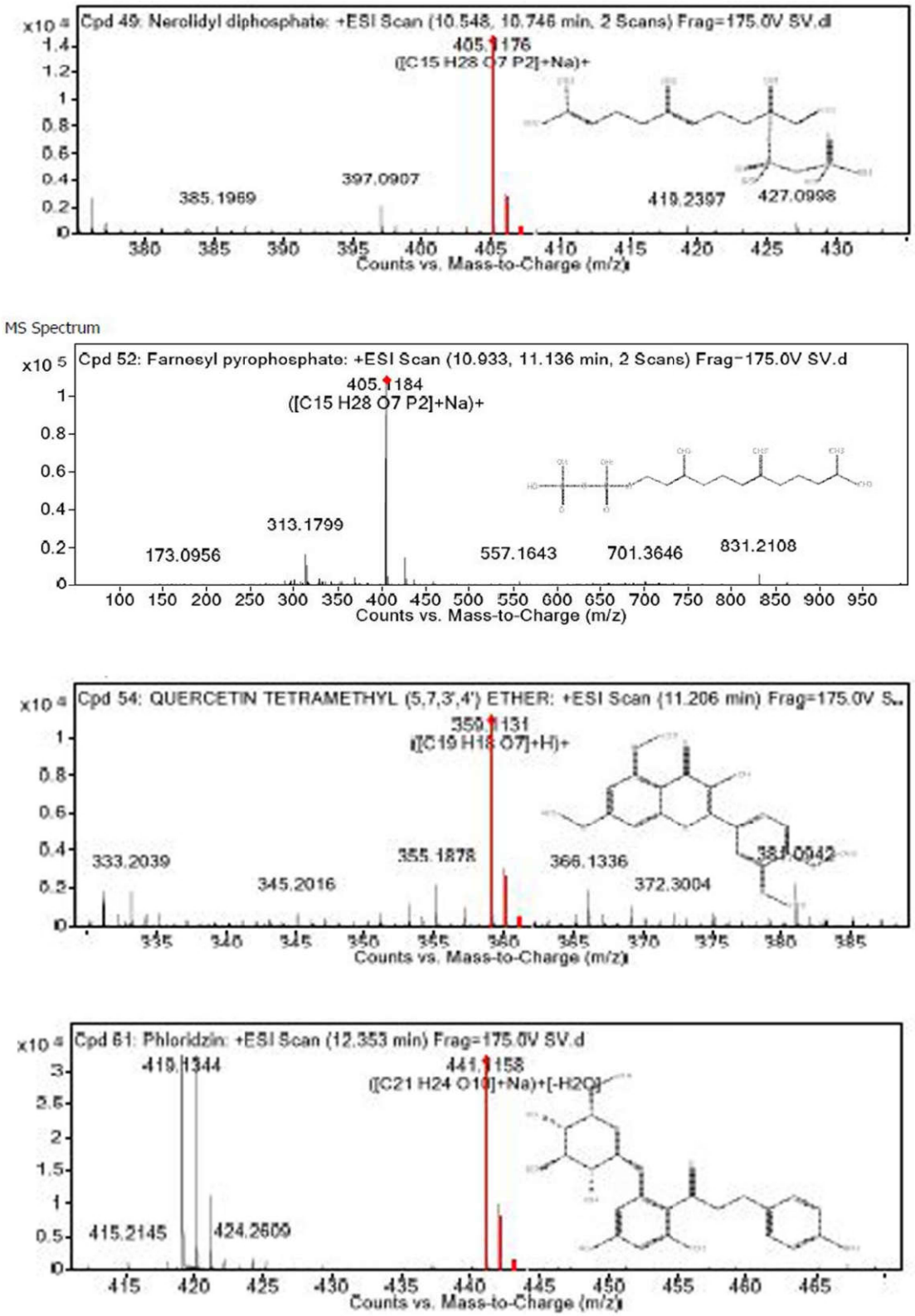


Figure 20 (iii): Mass spectra of compounds detected by HR-LC/MS analysis of methanolic extract of selected species of *Strobilanthes*

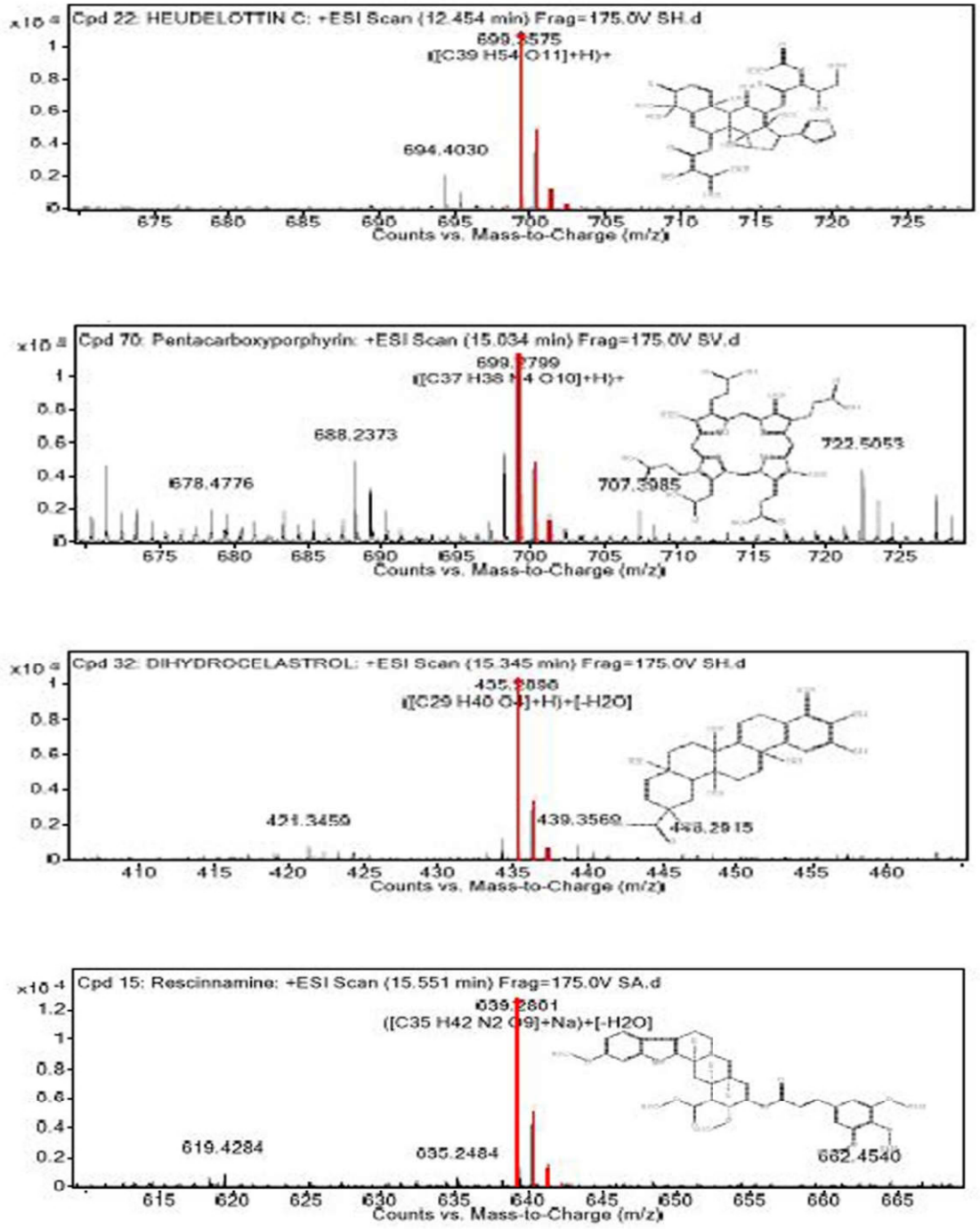


Figure 20 (iv): Mass spectra of compounds detected by HR-LC/MS analysis of methanolic extract of selected species of *Strobilanthes*

Table 18: Compounds detected from the GC/MS analysis of *Strobilanthes species*

Sl. No.	RT	Compound	Class	Peak area (%)		
				SA	SH	SV
1.	6.601	Heptanoic acid	Fatty acid	-	0.61	-
2.	6.735	2- Butoxypentane	Alkane	1.20	-	-
3.	7.535	2-Hexenoic acid	Fatty acid	-	0.94	-
4.	9.967	3-Acetyloxolan-2-one	Ketone	1.15	-	-
5.	11.904	5-Hydroxymethylfurfural	Furan	20.68	-	-
6.	12.503	Neodecanoic acid	Carboxylic acid	0.47	-	-
7.	17.325	Lauric acid	Fatty acid	1.78	-	-
8.	17.623	Neophytadiene	Terpene	2.74	4.42	0.46
9.	17.695	6,10,14-trimethyl -2-pentadecanone	Ketone	-	-	1.29
10.	17.968	β-Elemenone	Terpene	0.97	-	-
11.	18.167	Dodecanoic acid	Fatty acid	-	0.44	-
12.	18.740	Methyl palmitate	Fatty acid methyl ester	-	-	7.25
13.	19.996	Longiverbenone	Terpene	-	0.52	-
14.	20.152	Oleic acid	Fatty acid	2.63	-	-
15.	20.179	Palmitic acid	Fatty acid	9.34	8.39	0.51
16.	20.395	Loliolide	Terpene	0.50	-	-

17.	20.685	Khusimone	Ketone	2.13	-	-
18.	20.767	8, 11-Octadecadienoic acid methyl ester	Ester	-	-	1.59
19.	20.842	3,6-Octadecadienoic acid	Ester	-	-	12.92
20.	20.995	Phytol	Diterpene alcohol	6.78	13.68	2.22
21.	21.33	Decanoic acid	Fatty acid	-	0.43	-
22.	22.124	Flavone 4'-OH,5-OH,7-di-O-glucoside	Isoflavonoid	-	-	0.301
23.	22.411	Stearic acid	Fatty acid	-	-	0.505
24.	22.451	Hexahydrofarnesyl acetone	Ketone	-	0.55	-
25.	22.533	Tricyclo[20.8.0.0(7,16)]triacontane, 1(22),7(16)-diepoxy	Hydrocarbon	-	-	0.239
26.	22.813	Allyl stearate	Ester	-	-	0.647
27.	23.464	Camphol aldehyde	Terpene aldehyde	-	-	1.247
28.	24.029	Murolan-3,9(11)-diene-10-peroxy	Hydrocarbon	-	-	7.829
29.	24.384	Methyl linolenate	Ester	2.56	-	-
30.	24.891	α-Linolenic acid	Fatty acid	32.19	-	-
31.	25.113	Octadecanoic acid	Fatty acid	1.43	-	-
32.	26.392	Dihomo-γ-linolenic acid	Fatty acid	0.54	-	-
33.	27.046	3-butylcycloheptanone	Ketone	0.67	-	-
34.	28.908	Methyl docosanoate	Fatty acid methyl ester	-	0.90	-
35.	29.084	Squalene	Triterpene	0.19	32.75	8.79
36.	29.520	Lupeol trimethylsilyl ether	Ether	-	-	1.89

37.	30.370	Squalene oxide	Triterpene	-	-	0.58
38.	32.303	γ-Tocopherol	Vitamin	-	0.46	1.40
39.	32.446	δ-Tocotrienol	Vitamin	-	-	2.99
40.	35.794	Campesterol	Sterol	-	-	9.15
41.	35.884	3-β-Acetoxystigmasta-4,6,22-triene	Alkene	0.38	-	-
42.	36.401	Stigmasterol	Sterol	0.78	-	8.65
43.	37.225	Steviol	Terpene	-	0.17	-
44.	37.917	γ-Sitosterol	Sterol	1.87	14.25	19.90
45.	38.198	Ergost-5-en-3-ol	Sterol	0.40	2.44	-
46.	40.573	Vitamin E	Vitamin	-	1.61	-
47.	40.674	Lupeol	Terpenoid	8.64	10.43	9.64
48.	42.818	Stigmasta-5,22-dien-3-ol	Sterol	-	5.93	-
49.	47.621	Thunbergol	Diterpene alcohol	-	1.08	-

(**RT**: retention time; **SA**: *S. anamallaica*, **SH**: *S. hamiltoniana*, **SV**: *S. virendrakumarana*)

d) High-Resolution Liquid Chromatography/Mass Spectrometry (HR-LC/MS) analysis

The analysis to reveal non-volatile compounds of the three species procured about 21 potential phytocompounds with retention time that ranged from 0.98 to 20.98. The liquid chromatograms of the three species is shown in **Figures 17, 18 and 19**. The number of resolved compounds for *S. anamallaica, S. hamiltoniana* and *S. virendrakumarana* were 7, 10 and 12 respectively (**Table 19**). The mass spectra of individual compounds were shown in **Figure 20 (i-v**). Among them three were common in all the three species. They were swietenine, rescinnamine and dihydrogamboic acid. The molecular mass of these constituents in all the three species was within the limit of 212.095 to 698.35. The compounds having the least and highest molecular weight were harmine and heudelottin C. The compounds shared by *S. anamallaica* and *S. hamiltoniana* were punctaporin B and garcinolic acid. Campestanol and 1-hexadecanoyl-sn-glycerol were the constituents observed only in *S. anamallaica*. Whereas harmine, palaudine, dihydrocelastrol, heudelottin C and mesoporphyrin IX were only found in *S. hamiltoniana.*

PHARMACOGNOSTIC STUDIES

a) Powder microscopic studies

The powder microscopic images of the three species were evaluated for the pharmacognostic identification of them. Cystolith, calcium oxalate crystals and stone cells were common in all the species under consideration. In *S. anamallaica,* the powder showed the presence of pitted parenchyma cells, spiral vessels, fragments of tracheids, trichomes, fibres and prismatic crystals (**Plate 21**). The trichomes were multicellular with pointed end. In *S. hamiltoniana,* the resulted ones were fragments of fibre and trichomes, spiral vessels, starch grains and acicular crystals of calcium oxalate (**Plate 22**). Where as in *S. virendrakumarana,* the powder microscopic images resulted in fibres, vessels, multicellular trichomes, vascular traces of cell lamina, acicular crystals, prismatic crystals and lignified pith cells (**Plate 23**).

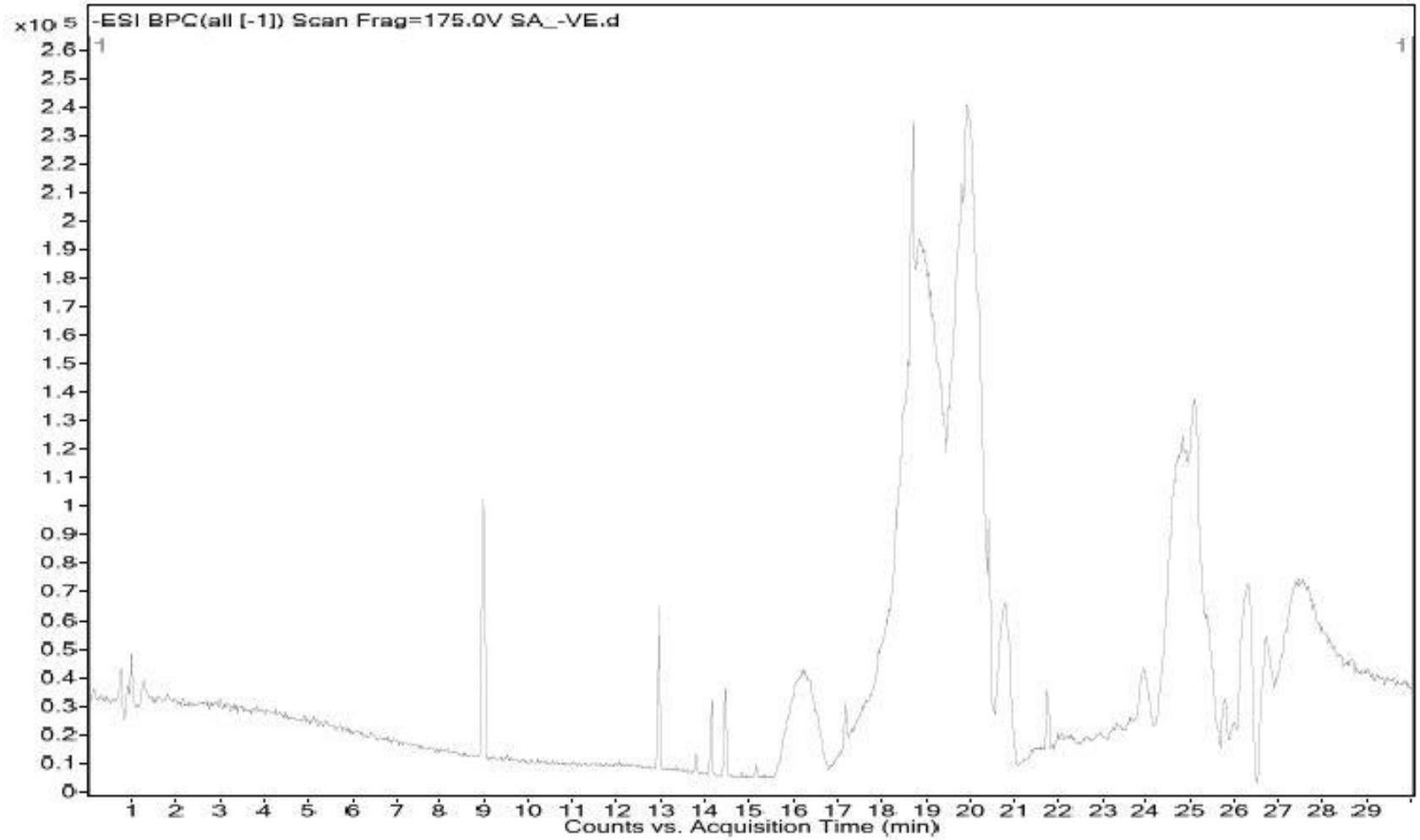


Figure 17: Liquid chromatogram of methanolic extract of *S. anamallaica*

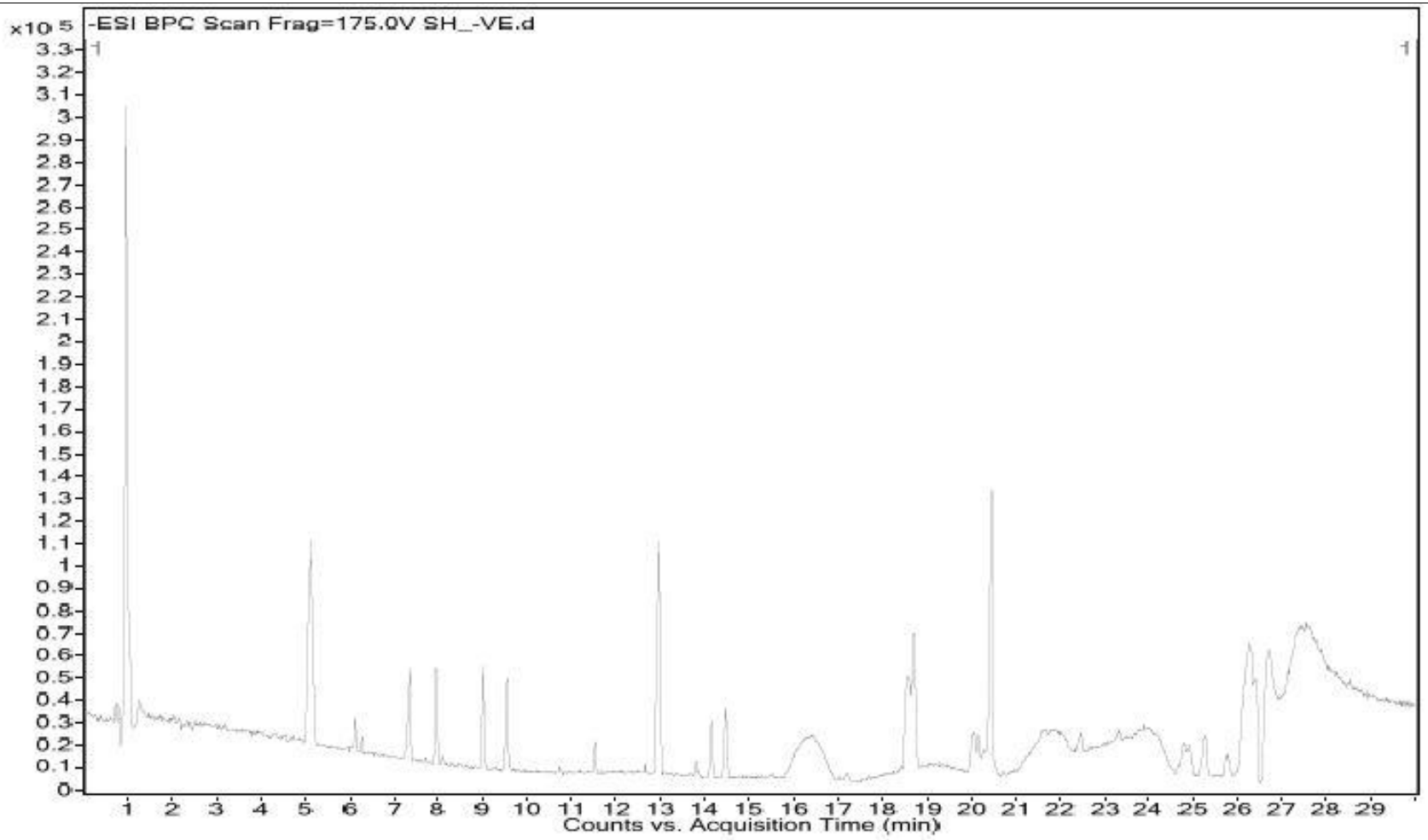


Figure 18: Liquid chromatogram of methanolic extract of *S. hamiltoniana*

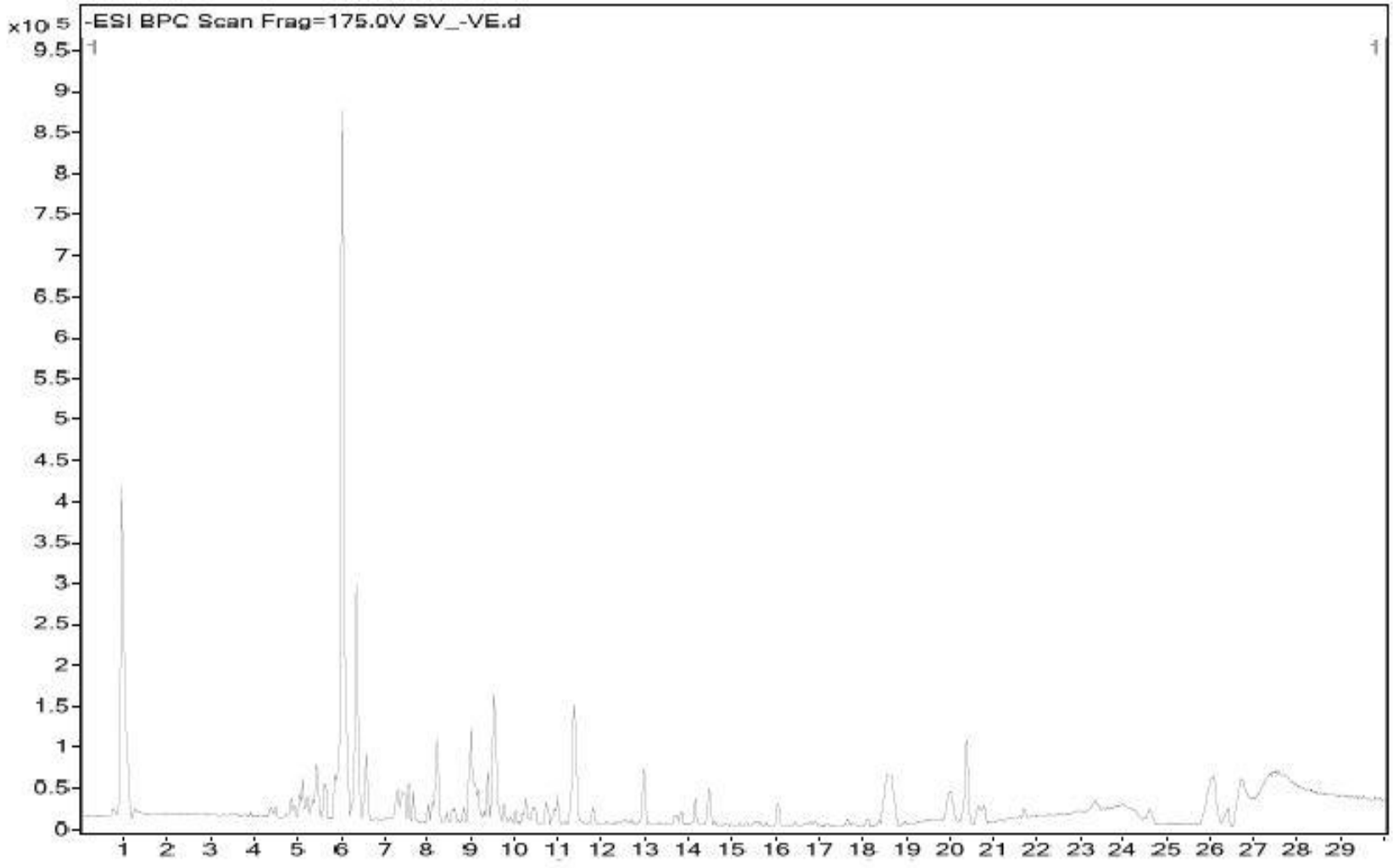


Figure 19: Liquid chromatogram of methanolic extract of *S. virendrakumarana*

Table 19: Compounds revealed from the HR-LC/MS analysis of *Strobilanthes species*

Sl No.	RT	Compound	Molecular mass	Class	SA	SH	SV
1.	0.98	Punctaporin B	252.1686	Terpene	+	+	-
2.	4.762	Harmine	212.095	Alkaloid	-	+	-
3.	5.247	11-Hydroxyiridodial glucoside Pentaacetate	556.2181	Terpene	-	-	+
4.	7.244	N-Desethyloxybutynin	329.1999	Benzenoid	-	-	+
5.	8.043	Epigallocatechin gallate	458.0824	Ester	-	-	+
6.	8.16	Palaudine	325.131	Alkaloid	-	+	-
7.	8.295	Aconitine	644.3186	Alkaloid	-	-	+
8.	8.98	Swietenine	568.274	Terpene	+	+	+
9.	10.666	Geraniol	382.1284	Terpene alcohol	-	-	+
10.	11.048	Farnesyl pyrophosphate	382.1291	Terpene	-	-	+
11.	11.226	3',4',5,7-Tetramethylquercetin	358.1058	Flavonoid	-	-	+
12.	12.369	Phloridzin	436.1372	Polyphenols	-	-	+
13.	12.474	Heudelottin C	698.35	Limonoids	-	+	-
14.	15.054	Pentacarboxyporphyrin	698.2734	Porphyrin	-	-	+
15.	15.366	Dihydrocelastrol	452.293	Terpene	-	+	-
16.	15.571	Rescinnamine	634.3034	Alkaloid	+	+	+
17.	16.746	Dihydrogambogic acid	630.308	Xanthonoid	+	+	+
18.	17.33	Campestanol	402.3873	Sterol	+	-	-
19.	17.97	1-Hexadecanoyl-sn-glycerol	330.277	Lipid	+	-	-
20.	18.465	Garcinolic acid	646.3023	Xanthonoid	+	+	-
21.	20.98	Mesoporphyrin IX	566.29	Porphyrin	-	+	-

(**RT**: retention time; **SA**: *S. anamallaica*, **SH**: *S. hamiltoniana*, **SV**: *S. virendrakumarana*; +: presence; -: absence)

b) ICP-MS analysis

In addition to the powder microscopic imaging, ICP-MS analysis of the shoot extract was also done. It gives the elemental composition of a sample in parts per billion. From the results, the composition of 18 elements in the three samples were identified. They were aluminium (Al), chromium (Cr), manganese (Mn), cobalt (Co), nickel (Ni), copper (Cu), zinc (Zn), arsenic (As), selenium (Se), strontium (Sr), molybdenum (Mo), barium (Ba), lithium (Li), magnesium (Mg), sodium (Na), potassium (K), iron (Fe) and calcium (Ca). In all the three, highest composition was recorded for K, Ca and Mg. The highest recorded value was for K in *S. anamallaica* with 18741.27 ppb and the lowest was for As in *S. hamiltoniana* with 0.02 ppb. The values were very low in Co, As, SE, MO and Li. The values of Cr, Ni, Zn and Fe were below 100 ppb. All the elements with their quantity in ppb are recorded (**Table 20**).

Table 20: ICP-MS analysis of selected species of *Strobilanthes*

Elements	*S. anamallaica*	*S. hamiltoniana*	*S. virendrakumarana*
Al	303.10	392.43	264.65
Cr	67.96	53.99	60.53
Mn	481.15	246.47	472.84
Co	1.90	1.01	2.16
Ni	32.01	25.04	31.52
Cu	127.94	47.37	52.79
Zn	11.10	13.14	23.23
As	0.023	0.02	0.04
Se	0.19	0.24	0.25
Sr	397.73	580.22	382.57
Mo	5.09	2.65	3.63
Ba	357.38	324.84	540.55
Li	0.33	-	0.45
Mg	16454.26	12619.10	16375.28
Na	534.88	543.44	517.36
K	18741.27	12292.16	13987.7
Fe	70.53	68.39	64.81
Ca	1107.9	932.42	1707.73

(Values are expressed in parts per billion)

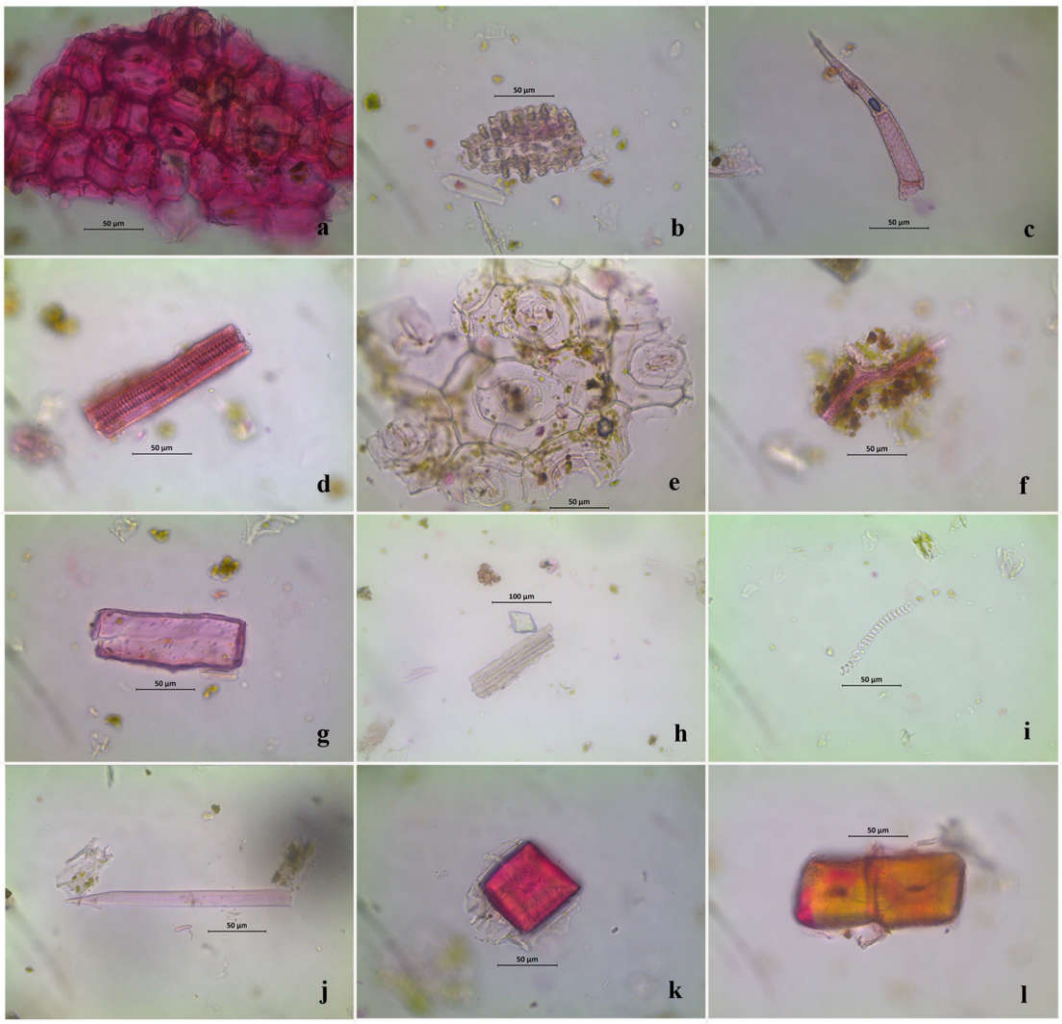


Plate 21: Powder microscopic images of *S. anamallaica* a. Epidermal cells of stem in surface view; **b.** Cystolith; **c.** Fragments of trichome; **d.** Fragments of tracheids; **e.** Lower epidermal cells of leaf; **f.** Cells of lamina with vascular traces; **g.** Pitted parenchyma cells; **h.** Prismatic crystals of calcium oxalate; **i.** Spiral vessel; **j.** Fibre; **k-l.** Stone cells

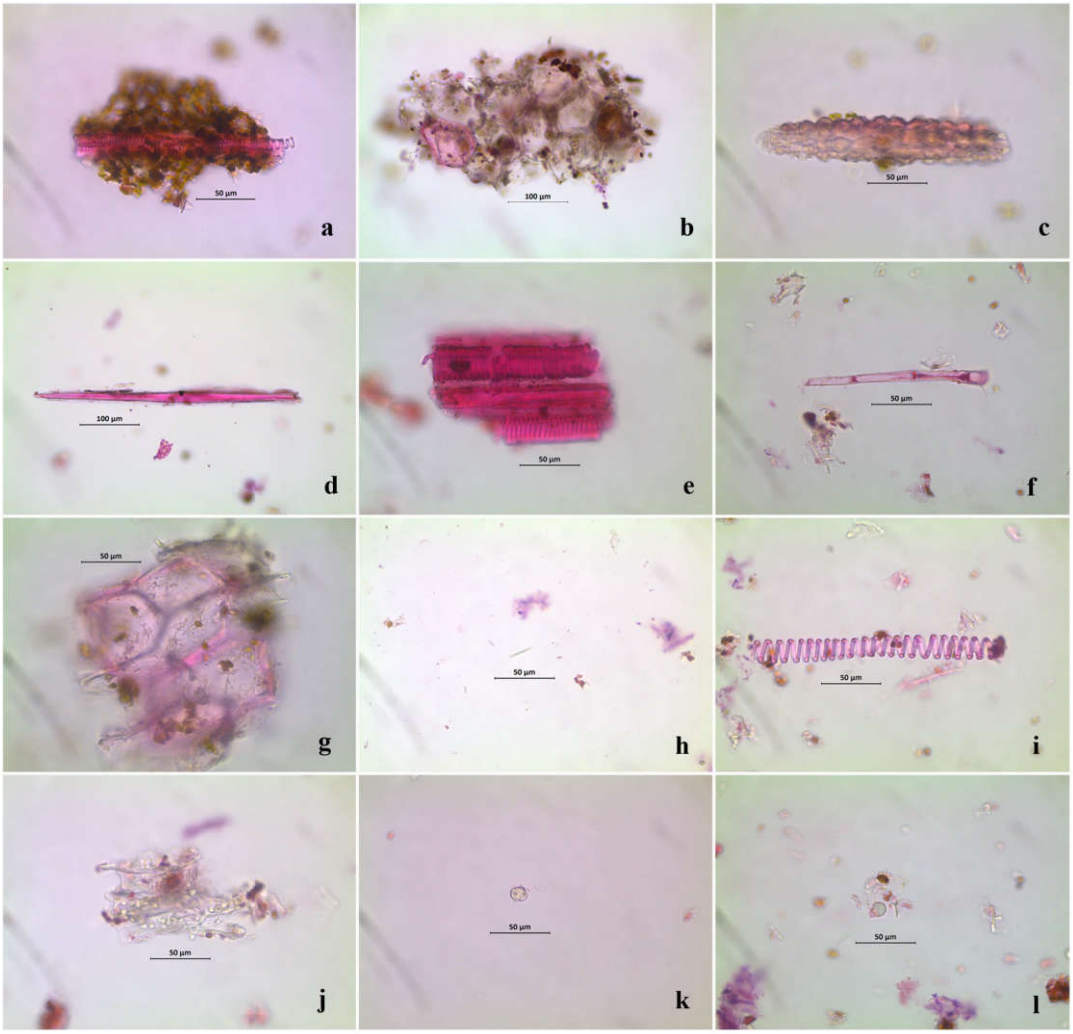


Plate 22: Powder microscopic images of *S. hamiltoniana* a. Cells of lamina with vessel fragments; **b.** Cortical cells of the stem; **c.** Cystolith; **d.** Fibre; **e.** Fragments of fibres and vessels; **f.** Fragments of multicellular trichome; **g.** Fragments of lignified cells of pith with acicular crystals of calcium oxalate; **h.** Acicular crystal; **i.** Spiral vessel; **j.** Parenchyma cells with starch grains; **k-l.** Starch grains

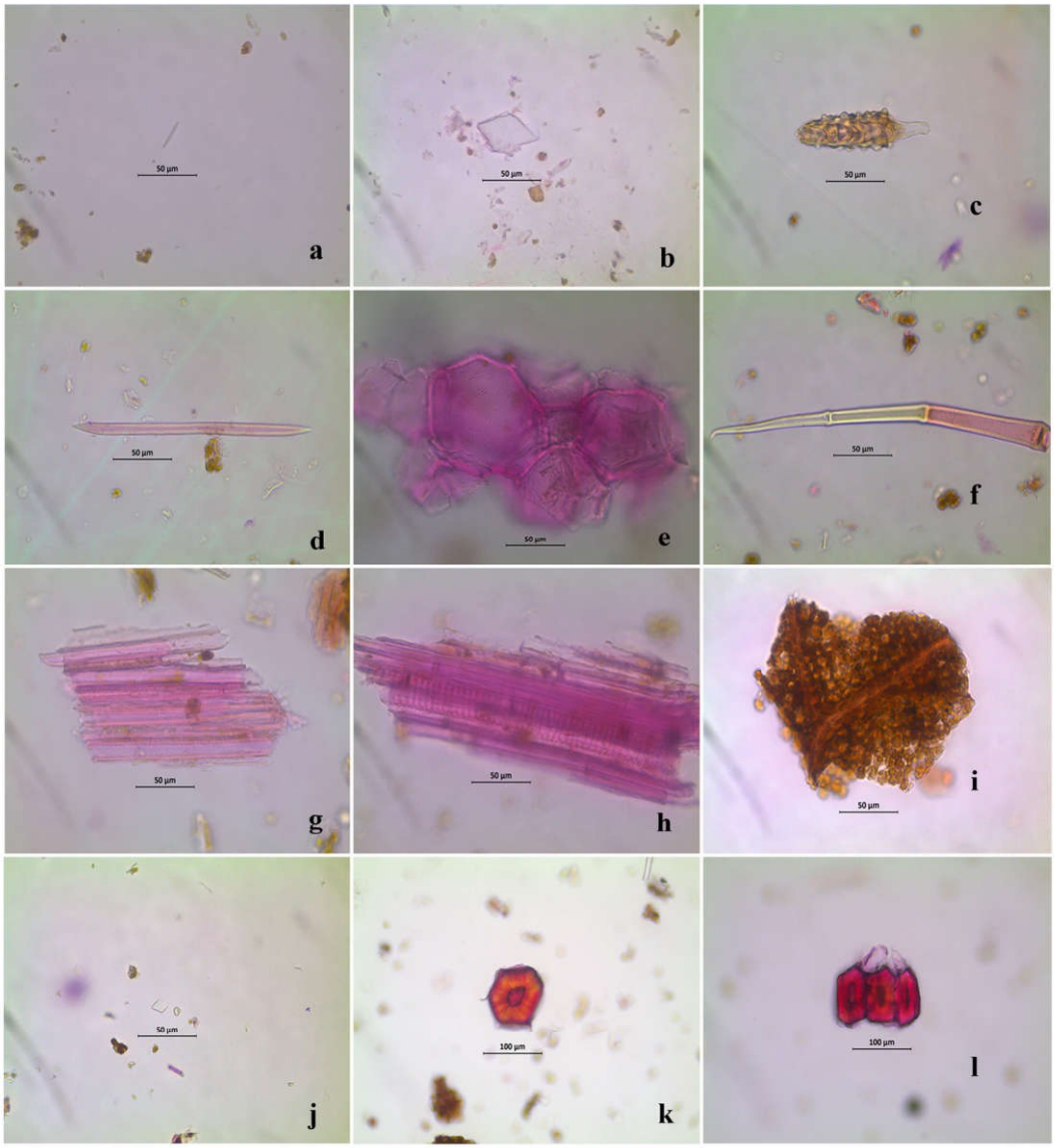


Plate 23: Powder microscopic images of *S. virendrakumarana* a. Acicular crystal; **b.** Crystal of calcium oxalate; **c.** Cystolith; **d.** Fibre; **e.** Fragments of lignified pith cells; **f.** Fragment of multicellular trichome; **g.** Fragments of tracheids and fibres; **h.** Fragments of vessels and fibres; **i.** Cells of lamina with vascular traces; **j.** Prismatic crystal; **k-l.** Stone cells

PHASE III - BIOACTIVITIES

A. Free radical scavenging activity

From the word itself it conveys the functional duty as a bodyguard to the oxidative stress caused by the oxidants. The cumulative effect caused by reactive oxygen species (ROS) / free radicals can be scavenged using antioxidant property. Their screening in plant and plant derived compounds requires proper methods & need to focus on the kinetics of their reaction. Each antioxidant assays have its own chemical principle to trap the free radical and convert them into visible change in the assay system. The three species of *Strobilanthes* were screened for DPPH radical scavenging assay, hydroxyl radical scavenging assay, superoxide free radical scavenging assay and reducing power activity.

1,1-diphenyl-2-picryl hydrazyl (DPPH) is having a pink colour which on scavenging leads to yellow. For this assay system, the concentrations of samples used were 12.5, 25, 50, 100 and 200 µg/mL. The concentration of gallic acid which is taken as standard (**Figure 21**) was also taken in accordance with the test sample. The discoloration intensity indicates the trapping potential of antioxidants in the extract. The concentration at which 50 percentage of potential scavenging has been effected is known as IC_{50} (**Table 21**). The IC_{50} values of all the extracts were within the concentrations selected. The standard showed 38.42 µg/mL as IC_{50}. The IC_{50} values of plant extracts such as *S. anamallaica, S. hamiltoniana* and *S. virendrakumarana* were 144.45, 172.01 and 39.09 µg/mL respectively (**Figure 22**). The IC_{50} value of *S. virendrakumarana* was least and the maximum percentage of inhibition (70.22%) was shown at 200 µg/mL.

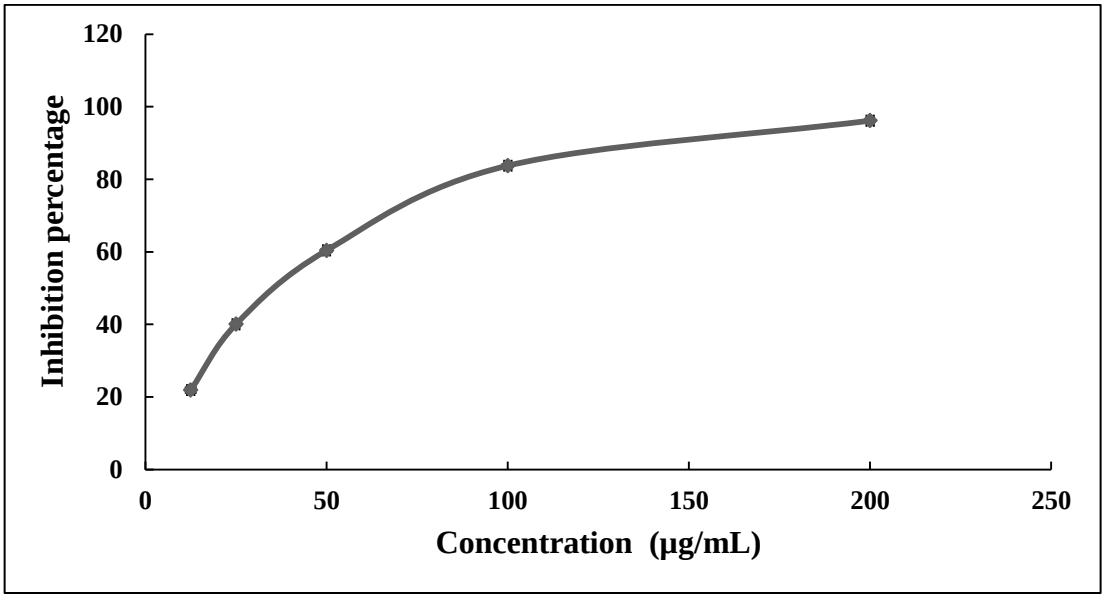


Figure 21: *In vitro* DPPH scavenging activity of the standard ascorbic acid

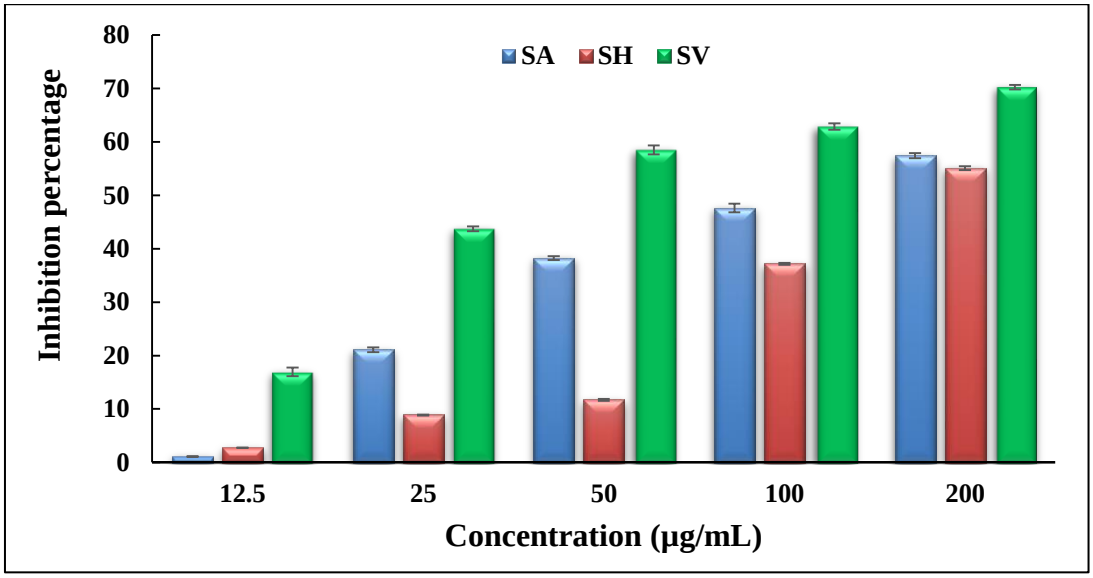


Figure 22: *In vitro* DPPH scavenging activity of selected species of *Strobilanthes*: SA - *Strobilanthes anamallaica*; SH - *Strobilanthes hamiltoniana*; SV - *Strobilanthes virendrakumarana*.

Hydroxyl radical scavenging assay system generates hydroxyl free radicals in order to catch the antioxidants within the sample. The reaction mixture follows the fenton reaction. The standard (gallic acid) and plant samples were taken in concentrations of 125, 250, 500, 1000 and 2000 µg/mL

(**Figure 23**). The IC_{50} values of the standard, *S. anamallaica, S. hamiltoniana* and *S. virendrakumarana* were found to be 477.49, 432.82, 368.23 and 375.24 µg/mL respectively. The maximum percentage of inhibition was for 2000 µg/mL of *S. virendrakumarana* extract. That is 76.72 ± 0.41 µg/mL. All the IC_{50} values were within the third concentration selected. Hence all the species shows high protecting activity (**Figure 24**).

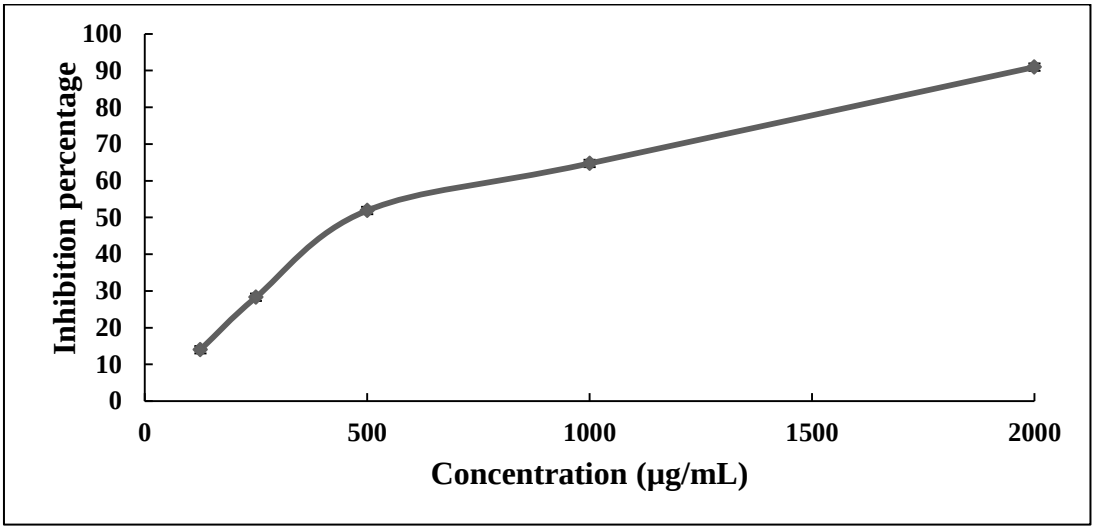


Figure 23: *In vitro* hydroxyl radical scavenging activity of the standard gallic acid

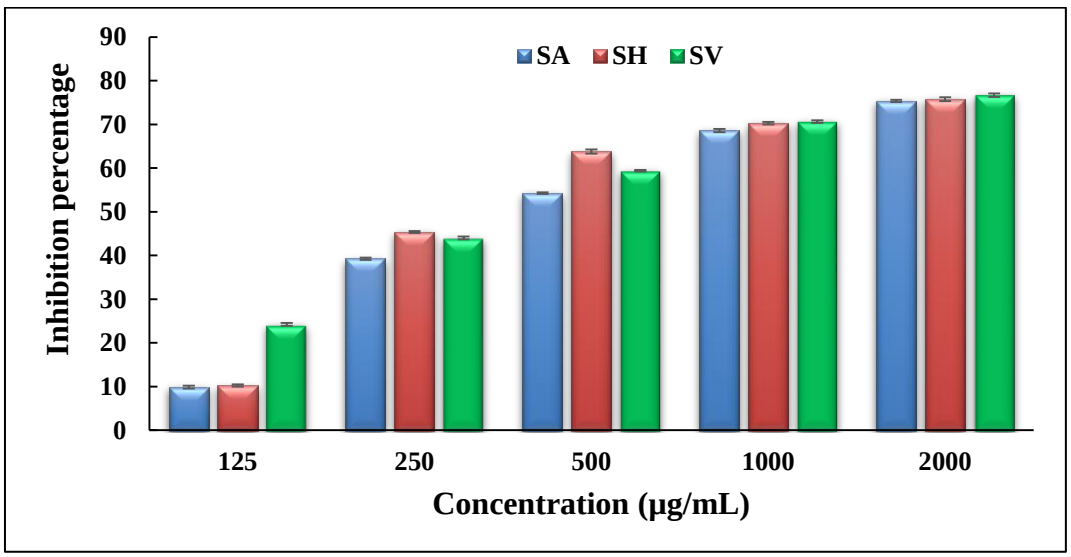


Figure 24: *In vitro* hydroxyl radical scavenging activity of selected species of *Strobilanthes*: SA - *Strobilanthes anamallaica*; SH - *Strobilanthes hamiltoniana*; SV - *Strobilanthes virendrakumarana*.

The standard used in superoxide free radical scavenging activity was ascorbic acid (**Figure 25**). The selected concentrations for experimental study were 125, 250, 500, 1000 and 2000 µg/mL. The reaction system of riboflavin-NADH superoxide anions is produced by oxidation of NADH. These anions were assayed by the blue formazan product formed as a result of NBT reduction. The IC_{50} values were high while comparing with other antioxidant assays performed. They were 283.48, 754.58, 931.33 and 749.47 µg/mL for standard, *S. anamallaica, S. hamiltoniana and S. virendrakumarana* respectively. Among the test samples highest inhibition was shown by *S. hamiltoniana* (76.48 ± 0.29) at 2000 µg/mL (**Figure 26**).

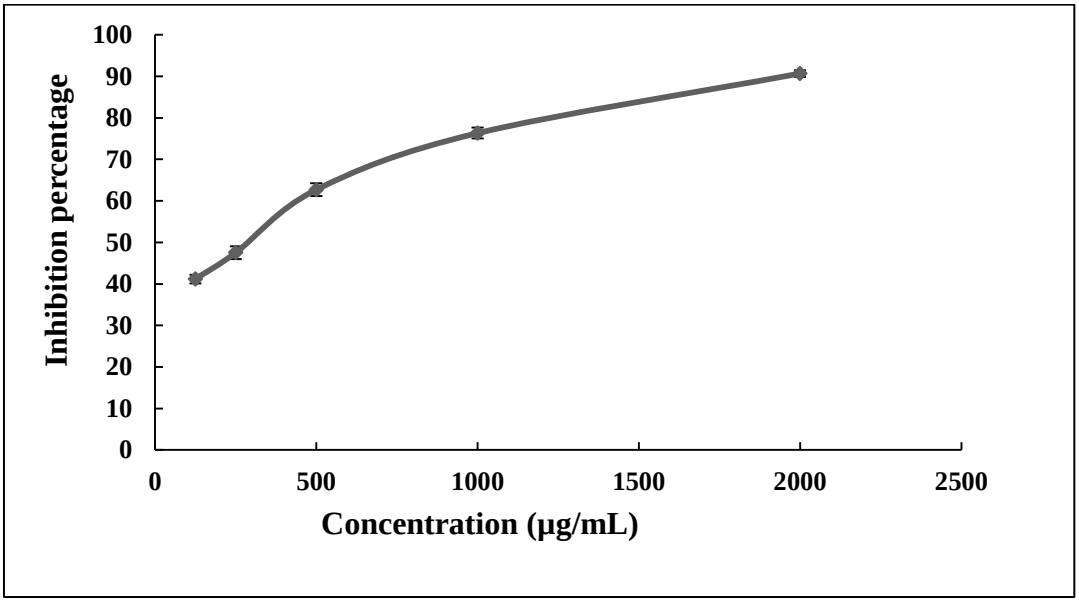


Figure 25: *In vitro* superoxide radical scavenging activity of the standard ascorbic acid

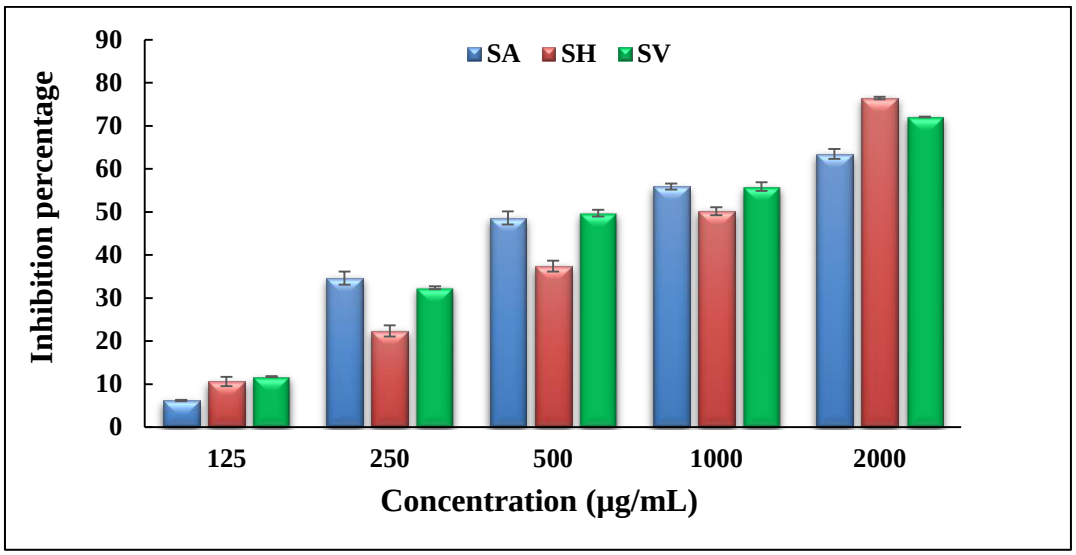


Figure 26: *In vitro* superoxide radical scavenging activity of selected species of *Strobilanthes*: SA - *Strobilanthes anamallaica*; SH - *Strobilanthes hamiltoniana*; SV - *Strobilanthes virendrakumarana*.

Reducing power of extract was determined using the concentrations such as 125, 250, 500, 1000 and 2000 µg/mL for all the samples. Quercetin was the standard used. In contradiction to other assays, in reducing power assay absorbance itself is taken as an indicator of inhibition. The absorbance increase for standard was from 1.04 ± 0.04 to 2.44 ± 0.05 (**Figure 27**). The initial absorbance for the concentration *viz.*, 125 µg/mL was almost the same for all the *Strobilanthes* species. The absorbance increased to 0.22 ± 0.05, 0.28 ± 0.01 and 0.28 ± 0.14 for *S. anamallaica, S. hamiltoniana and S. virendrakumarana* respectively (**Figure 28**).

Table 21: Inhibition concentration in antioxidant assays

Plant	IC_{50} Value (µg/mL)		
	DPPH radical scavenging assay	**Hydroxyl radical scavenging activity**	**Super oxide free radical scavenging activity**
S. anamallaica	144.45 ± 2.28	432.82 ± 2.03	754.58 ± 2.05
S. hamiltoniana	172.01 ± 2.08	368.23 ± 3.23	931.33 ± 2.32
S. virendrakumarana	39.09 ± 1.30	375.24 ± 2.03	749.47 ± 2.04
Standard	38.42 ± 2.32	477.49 ± 2.36	283.48 ± 2.62

(All values are expressed in mean ± SE)

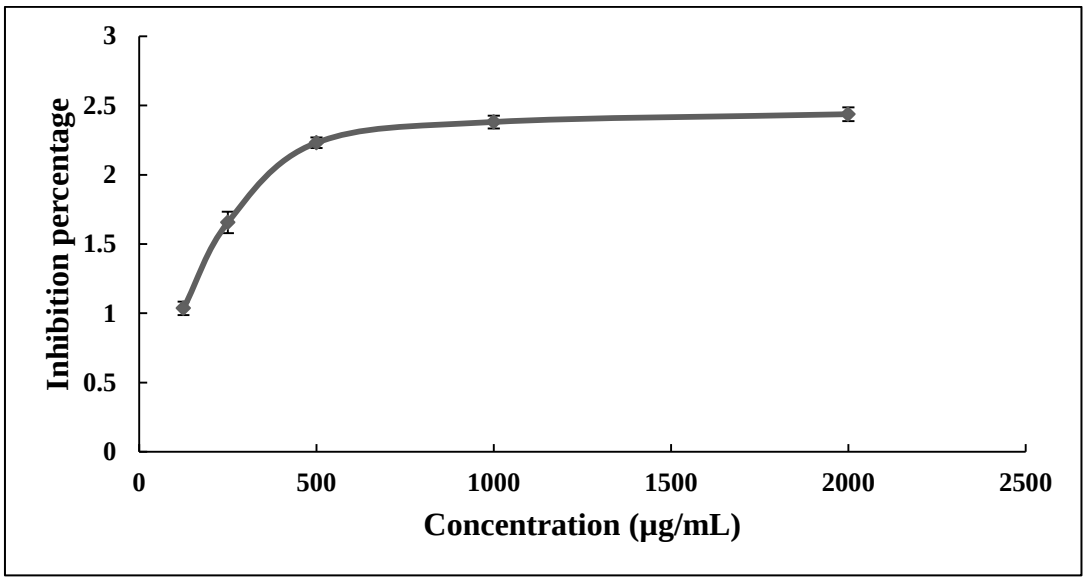


Figure 27: *In vitro* reducing power activity of the standard quercetin

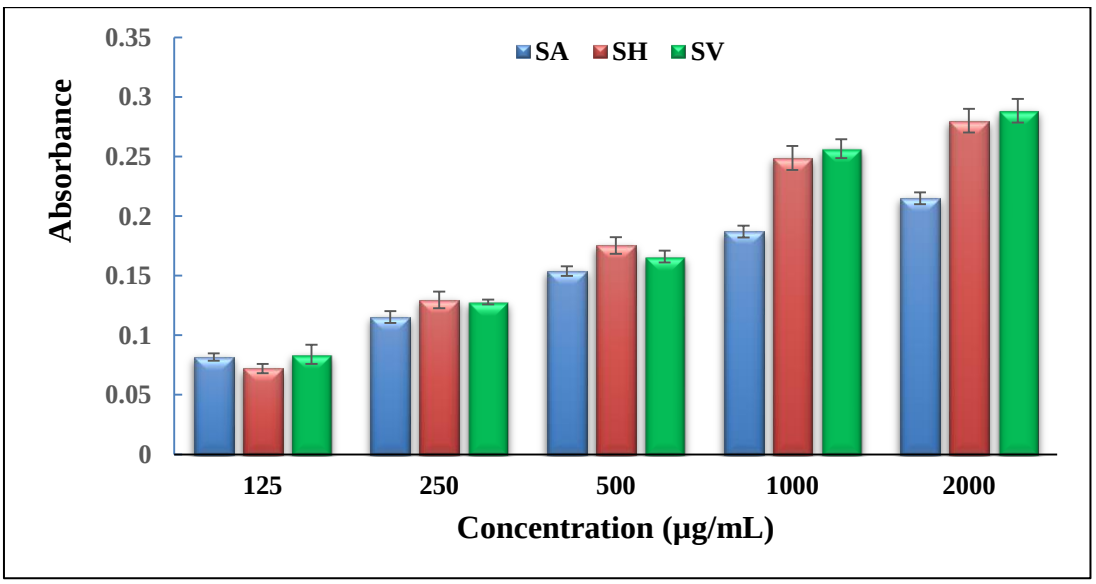


Figure 28: *In vitro* reducing power activity of selected species of *Strobilanthes*: SA - *Strobilanthes anamallaica*; SH - *Strobilanthes hamiltoniana*; SV - *Strobilanthes virendrakumarana*.

In DPPH and hydroxyl radical scavenging assays, maximum inhibition of free radicals was shown by the highest concentration of *S. virendrakumarana*. Moreover, *S. anamallaica* showed highest percentage of protection in superoxide free radical scavenging activity. A moderate activity was shown by all the three species under study in reducing power assay.

B. Cytotoxicity by *Allium cepa*

The methanolic shoot extract of the selected species of *Strobilanthes* brought out divergent aberrations in *Allium cepa* assay which include both clastogenic and non-clastogenic ones. The normal stages of *A. cepa* (**Plate 24**) were disturbed in presence of the methanolic extract of *Strobilanthes*. The bulbs were treated for 24 h in all the three methanolic extracts. A positive correlation between extract concentration and abnormality percentage was noticed. The mitotic index (MI) declined with respect to the rise in the concentration of the extract (**Figure 29 a**). MI revealed a hike in the negative control (78.47%) and was low with 30.51% in positive control. When comparing the species, the maximum value was recorded in 25 µg/mL of *S. anamallaica* and lowest in 100 µg/mL of *S. virendrakumarana*. The abnormality percentage ranges between 72.83% and 10.62% (**Figure 29 b**) and that of mitotic index was between 78.47% and 21.57%.

The major aberrations resulted in interphase and prophase were nuclear erosion, lesion, budding, fragmentation, giant cell showing formation of single lesion and breakage, cytoplasmic vacuolation, chromatin granules, unequal condensation *etc.* (**Plate 25, 26**). Number of aberrations were high in metaphase. These includes ball metaphase, lesion, chromosome erosion and vagrants, pole to pole metaphase, misorientation of chromosomes, chained metaphase, stellate metaphase, partial C-metaphase, somatic pairing at pole to pole metaphase, tropokinesis, sticky metaphase *etc.* (**Plate 27**). Likewise in anaphase also aberrations were elevated. Diagonal stathmo-anaphase, sticky anaphase, chromosome erosion and vagrants, unequal separation with bridges, shifting of microtubule organising centre *etc.* (**Plate 28**). In telophase and cytokinesis aberrations were low in number. These consist of misorientation after equatorial separation, early cell plate formation showing lesion and pulverized chromatin, misorientation of chromosome groups at cytokinesis showing nuclear lesion and erosion *etc.* (**Plate 29**).

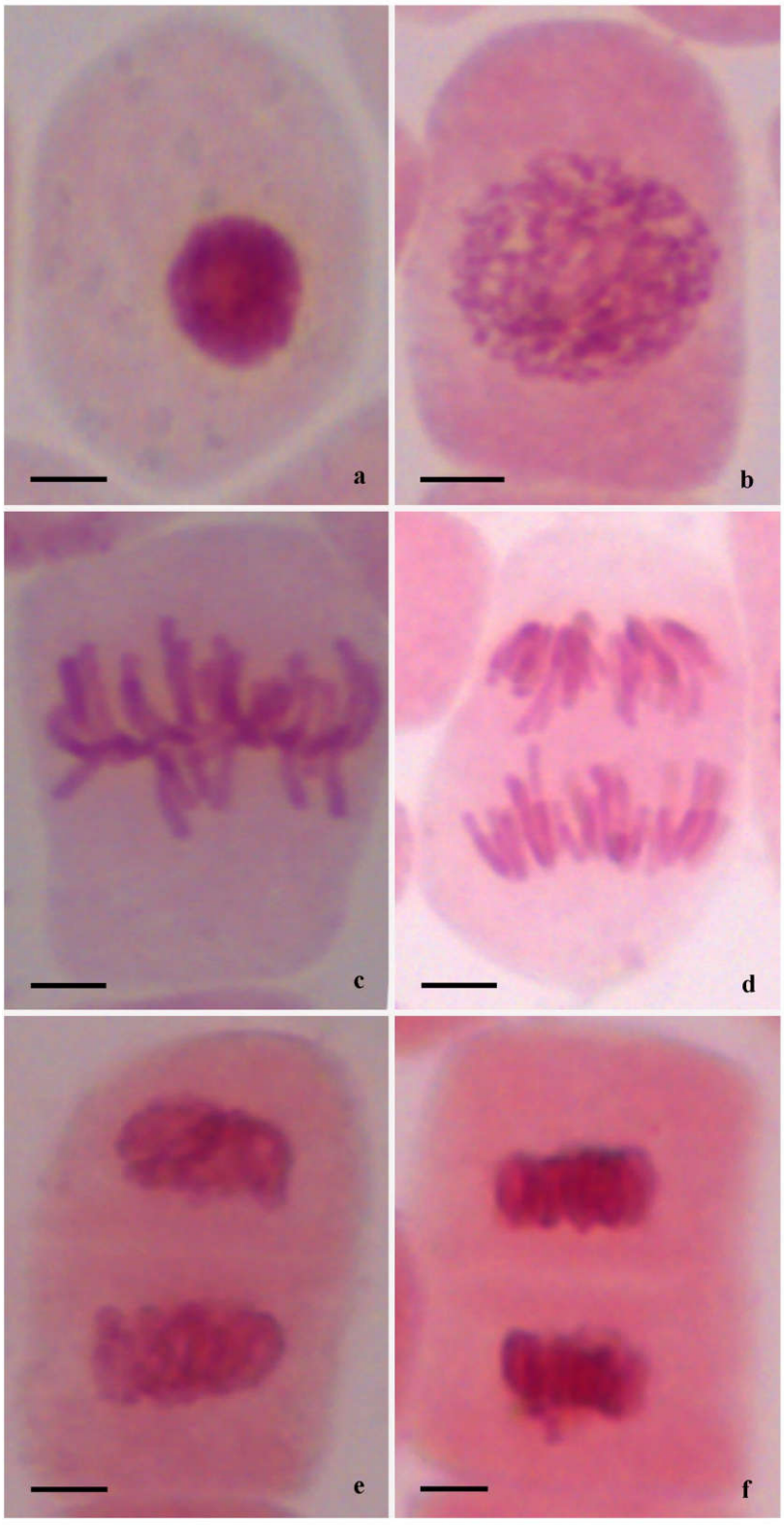


Plate 24: Normal stages of *Allium cepa* a. Interphase; **b.** Prophase; **c.** Metaphase; **d.** Anaphase; **e.** Telophase, **Bar - 10 µm**

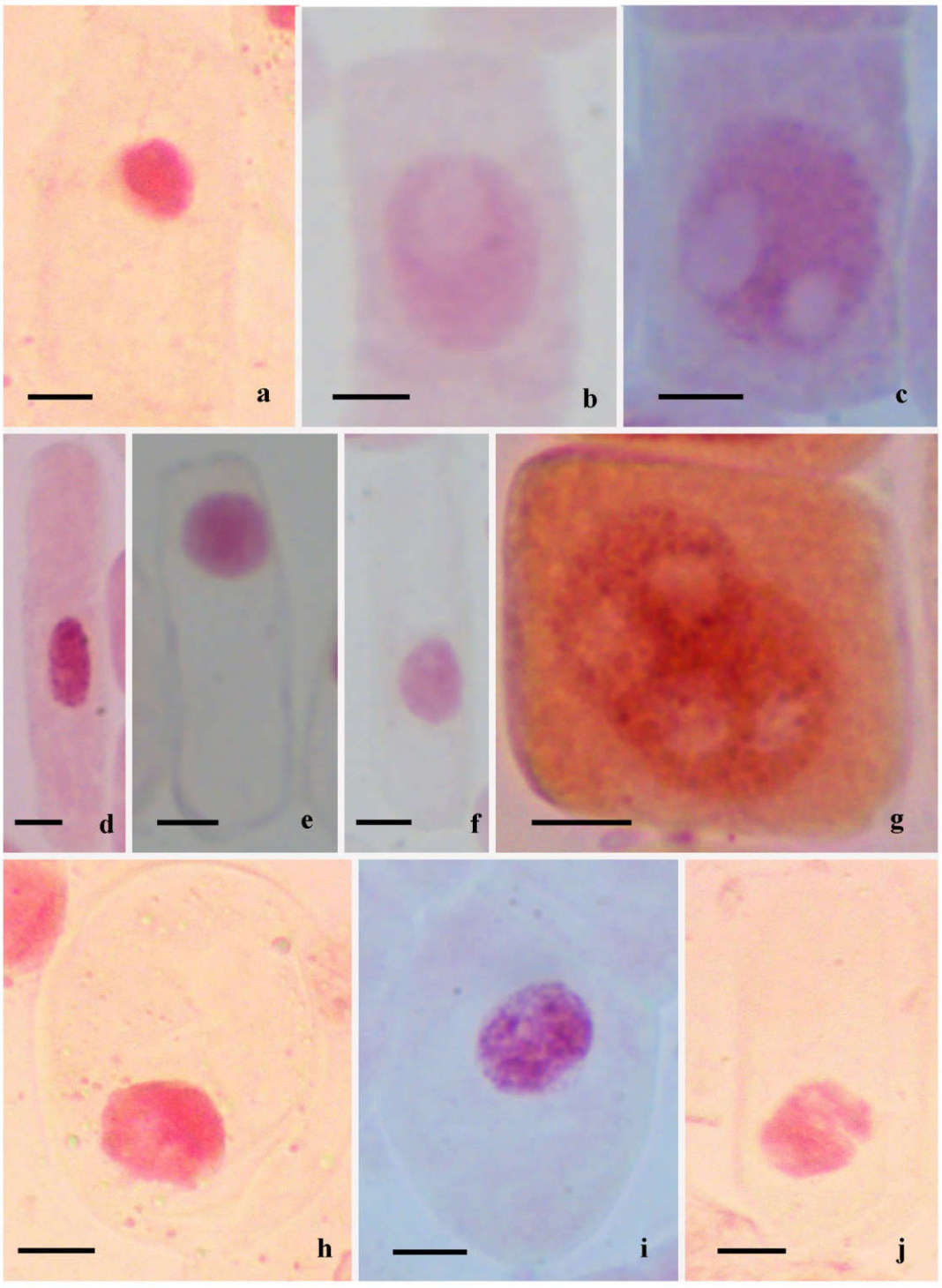


Plate 25: Chromosomal aberrations induced by the extracts of *Strobilanthes* species in *Allium cepa* at Interphase a. Nuclear erosion; **b.** Single nuclear lesion; **c.** Double nuclear lesion; **d.** Giant cell showing formation of single lesion; **e.** Cytoplasmic vacuolation; **f.** Giant cell showing nuclear breakage; **g.** Giant nucleus with multiple nuclear lesion; **h.** Nuclear budding; **i.** Chromatin granules; **j.** Nuclear fregmentation, **Bar - 10 µm**

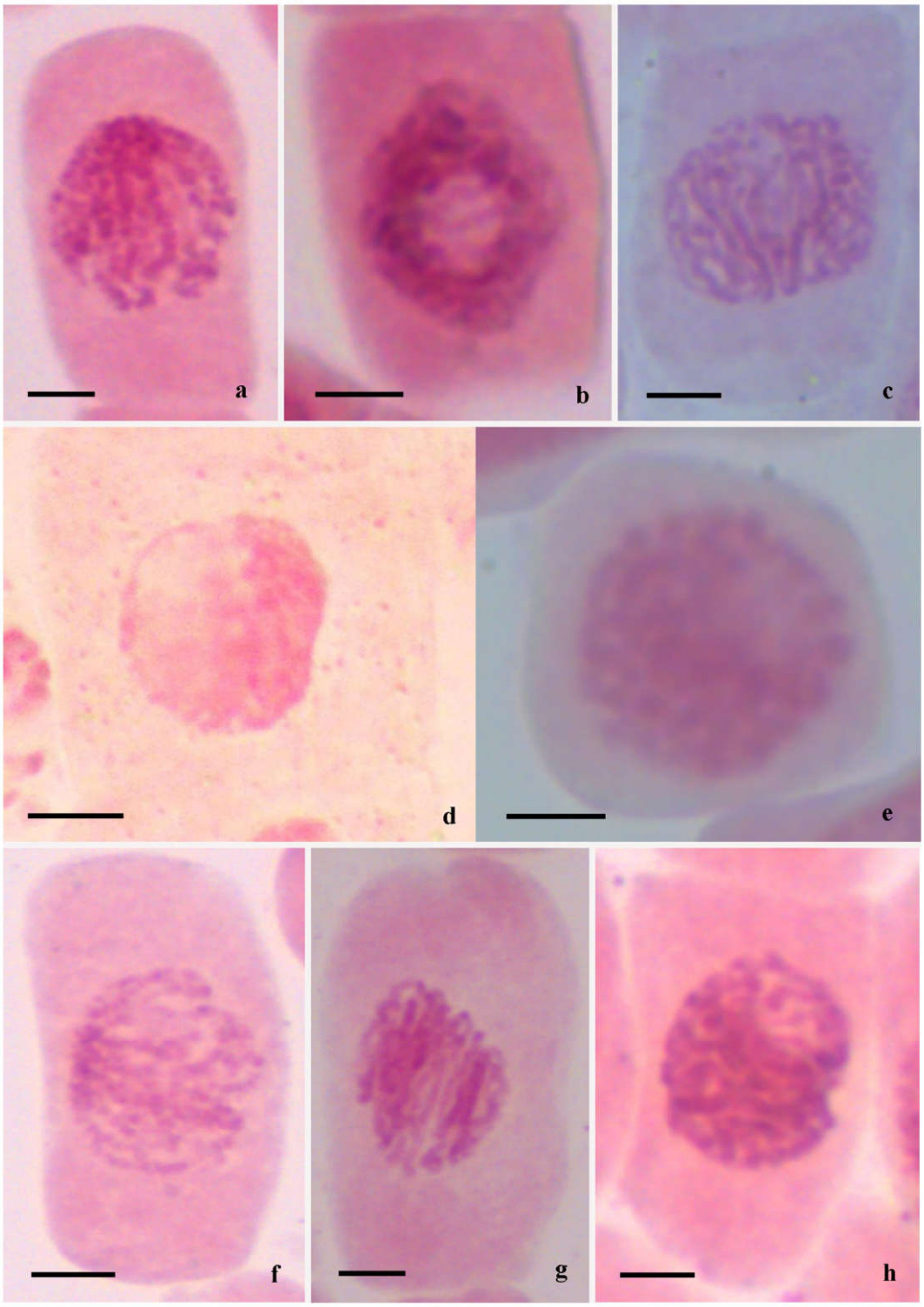


Plate 26: Chromosomal aberrations induced by the extracts of *Strobilanthes* species in *Allium cepa* at Prophase a. Chromatin erosion at late prophase; **b.** Single nuclear lesion; **c.** Chromatin erosion; **d.** Coagulated chromatin showing lesions; **e.** Formation of lesion; **f.** Pulverised chromatin; **g.** Unequal condensation; **h.** Lesion, **Bar - 10 µm**

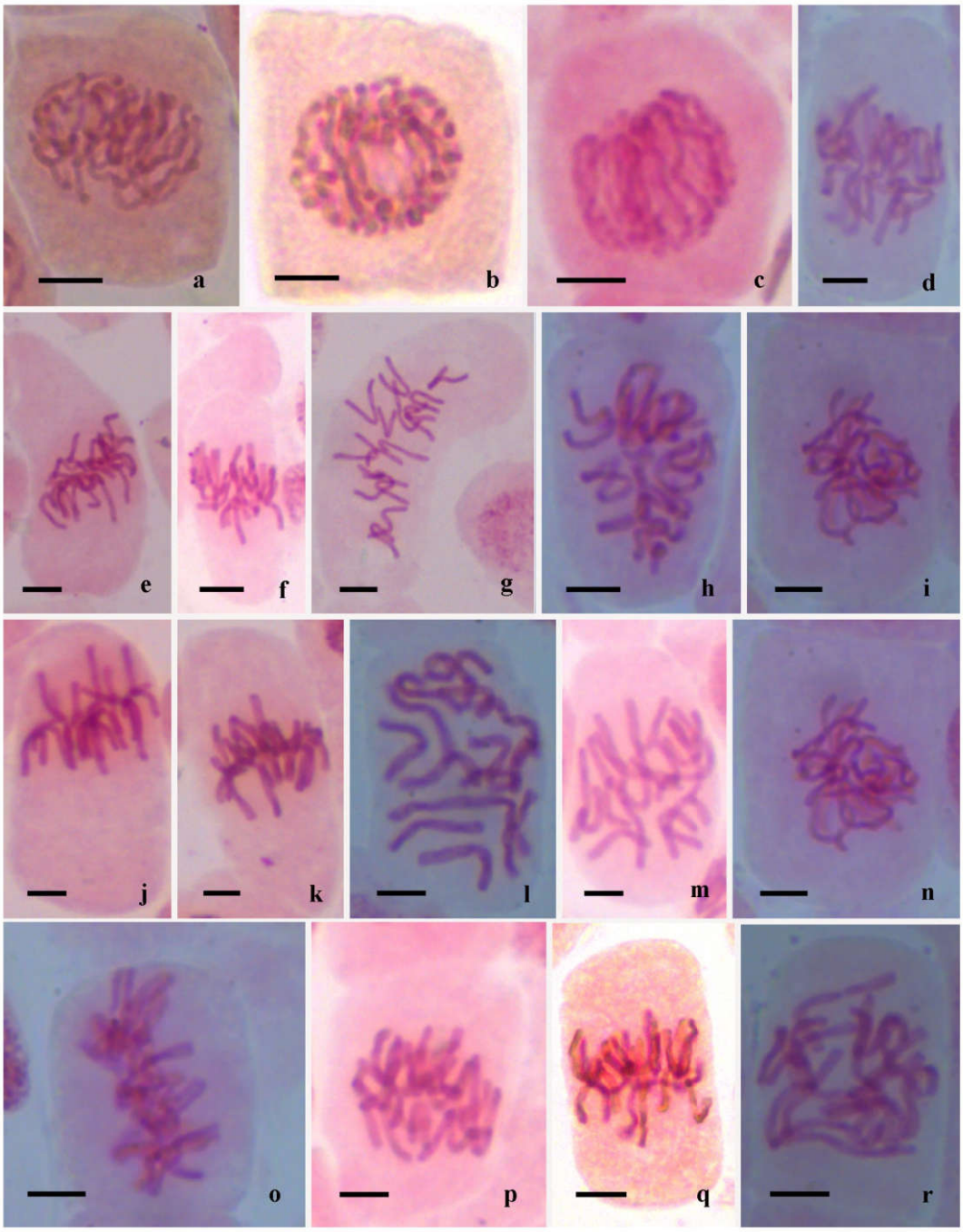


Plate 27: Chromosomal aberrations induced by the extracts of *Strobilanthes* species in *Allium cepa* at Metaphase a. Ball metaphase; **b.** Lesion; **c.** Pulverised ball metaphase; **d.** Chromosome erosion; **e.** Chromosome vagrants; **f.** Disturbed metaphase in a hyperploid cell; **g.** Pole to pole metaphase; **h.** Misorientation of chromosomes; **i.** Chained metaphase; **j.** Misorientation of stellate metaphase; **k.** Partial C-metaphase; **l.** Partial scattered C-metaphase; **m.** Scattered metaphase; **n.** Chained metaphase; **o.** Somatic pairing at pole to pole metaphase; **p.** Tropokinesis; **q.** Sticky metaphase; **r.** Sticky disturbed C-metaphase, **Bar - 10 µm**

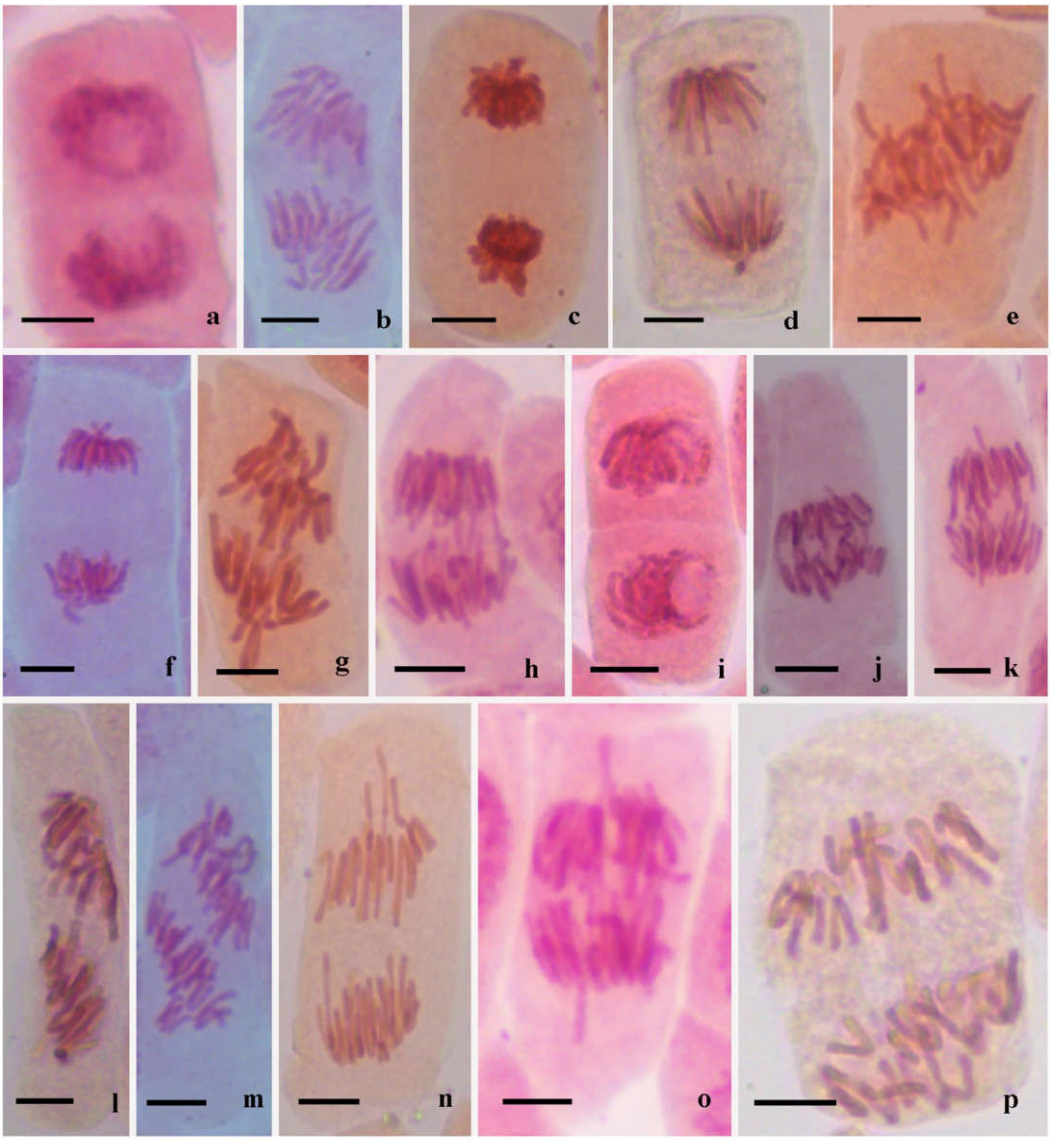


Plate 28: Chromosomal aberrations induced by the extracts of *Strobilanthes* species in *Allium cepa* at Anaphase a. Pulverised chromatin; **b.** Chromosome erosion; **c.** Coagulated anaphase; **d.** Sticky anaphase; **e.** Diagonal stathmo-anaphase; **f.** Chromosome vagrants; **g.** Diagonal anaphase showing vagrants and laggards; **h.** Multiple sticky bridges; **i.** Formation of lesion among daughter chromosome groups; **j.** Unequal separation showing multiple bridges; **k.** Unequal separation showing bridges; **l.** Shift in MTOC **m.** Diagonal unequal separation; **n.** Vagrant chromosomes; **o.** Sticky stathmo-anaphse; **p.** Scattered diagonal anaphase showing vagrants, **Bar - 10 µm**

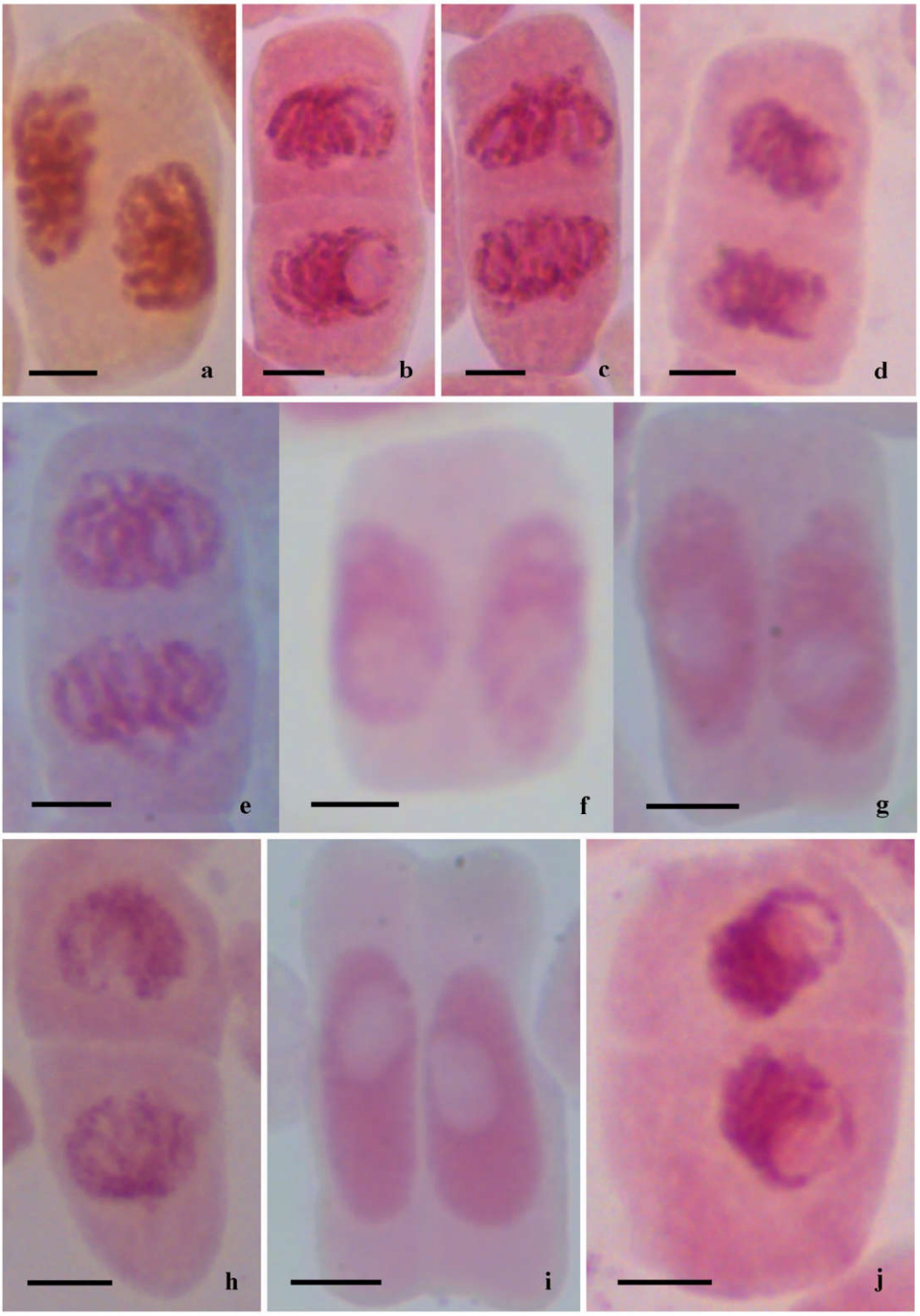


Plate 29: Chromosomal aberrations induced by the extracts of *Strobilanthes* species in *Allium cepa* at Telophase (a-d) and Cytokinesis (e-j) a. Misorientation after equatorial separation; **b.** Early cell plate formation showing lesion among daughter chromosome groups; **c.** Early cell plate formation showing pulverised chromosomes; **d.** Pulverised chromosomes; **e.** Chromatin erosion; **f.** Chromatin lesion in daughter nuclei after equatorial separation; **g.** Formation of multiple lesions in daughter nuclei after equatorial separation; **h.** Pulverised chromatin; **i.** Single lesions in daughter nuclei after equatorial separation **j.** Misorientation of chromosome groups at cytokinesis showing nuclear lesion and erosion, **Bar - 10 µm**

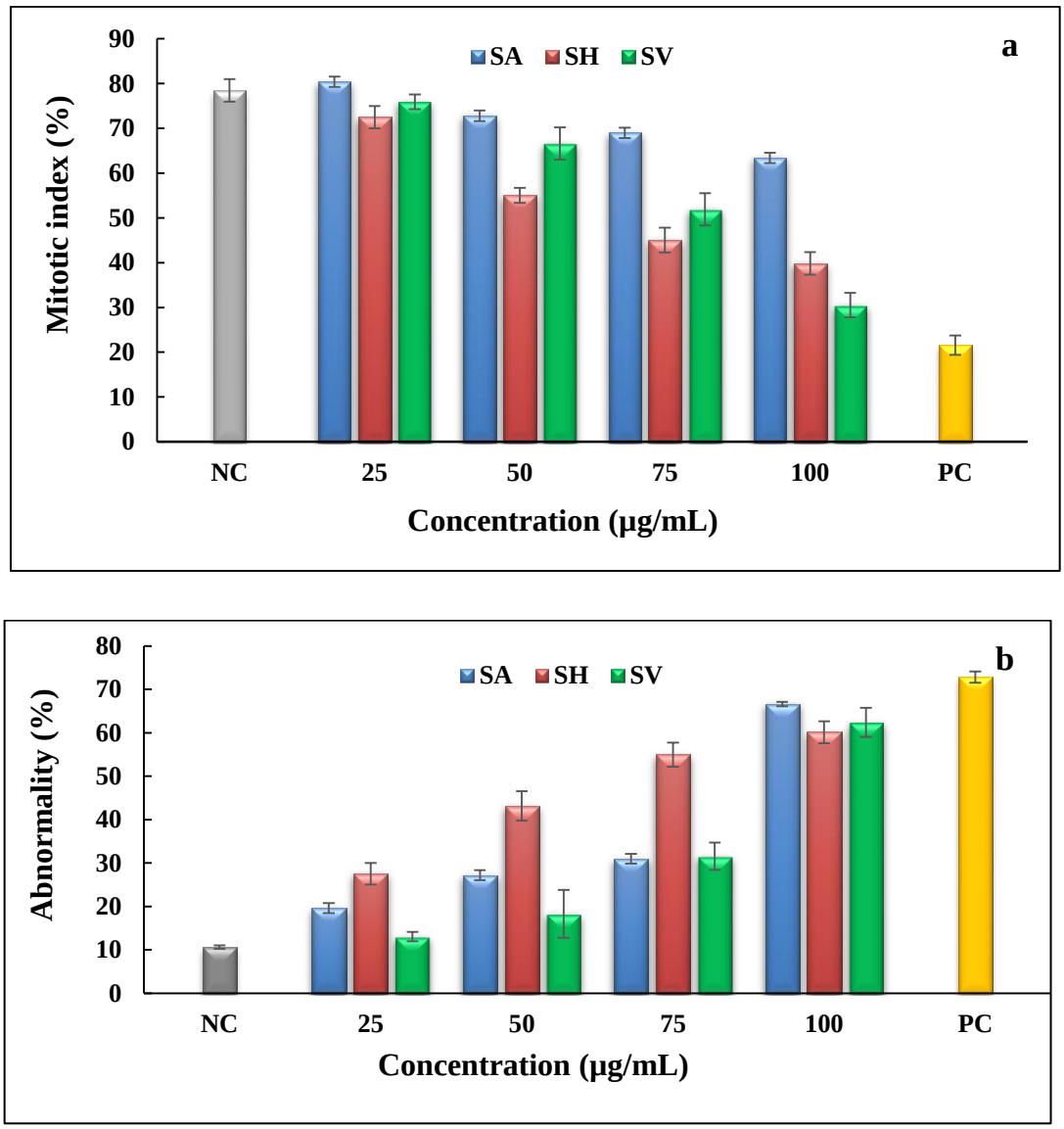


Figure 29: Cytotoxicity in *Allium cepa* by selected species of *Strobilanthes*: a. Mitotic index; b. Abnormality percentage, SA - *Strobilanthes anamallaica*; SH - *Strobilanthes hamiltoniana*; SV - *Strobilanthes virendrakumarana*; NC - negative control; PC – positive control

C. Antiproliferative efficacy of *Strobilanthes*

The Human Colorectal Adenocarcinoma cell line DLD-1 and normal cell line L929 were used for studying cytotoxicity of selected species of *Strobilanthes.* The cytotoxic potential of different concentrations of the extract was screened using MTT assay. The LC_{50} values in both cell lines were compared to conclude the most effective species for further studies.

i. *In vitro* anticancer activity using MTT assay in DLD1 cell line

To analyse the antiproliferative activity of selected species of *Strobilanthes* in DLD1 cell lines, different concentrations selected were 6.25, 12.5, 25, 50 and 100 µg/mL. It resulted in different types of aberrations within the cell line. They were apoptotic bodies, budding, cell shrinkage, condensed nuclei, membrane blebbing, nuclear fragmentation and echinoid spikes (**Plates 30, 31, 32**). The LC_{50} value of *S. virendrakumarana* was found to be a promising one. The LC_{50} values were tabulated (**Table 23**). The cell death characterisation of control and treated DLD1 cell lines were clearly visible under a microscope. With the help of a Software ED50 PLUS VT.O, LC_{50} values were calculated.

For all concentrations taken, absorbance was considered to measure the percentage of viable cells. The dose-dependent decrease in viable cells were exhibited by all the three species. At the lowest concentration (6.25 µg/mL), *S. hamiltoniana* acted as a better candidate. But the other two species showed more or less equal viability percentage and the decline was uniform for these species. For *S. anamallaica*, the viability percentage decreased from 95.07 ± 1.61 to 70.99 ± 1.57. At the highest concentration (100 µg/mL), both *S. hamiltoniana* and *S. virendrakumarana* showed almost equal viability percentage (**Figure 30**). This resemblance was reflected in their LC_{50} values also. The LC_{50} for *S. virendrakumarana* and *S. hamiltoniana* was 111.99 and 115.64 µg/mL. But the percentage of viability drop was highest in *S. virendrakumarana*. The value declined from 96.31 ± 1.63 to 56.07 ± 2.79. While analysing and comparing the viability percentage and LC_{50} values of all the three species, *S. virendrakumarana* seems to be a superior delegate among them.

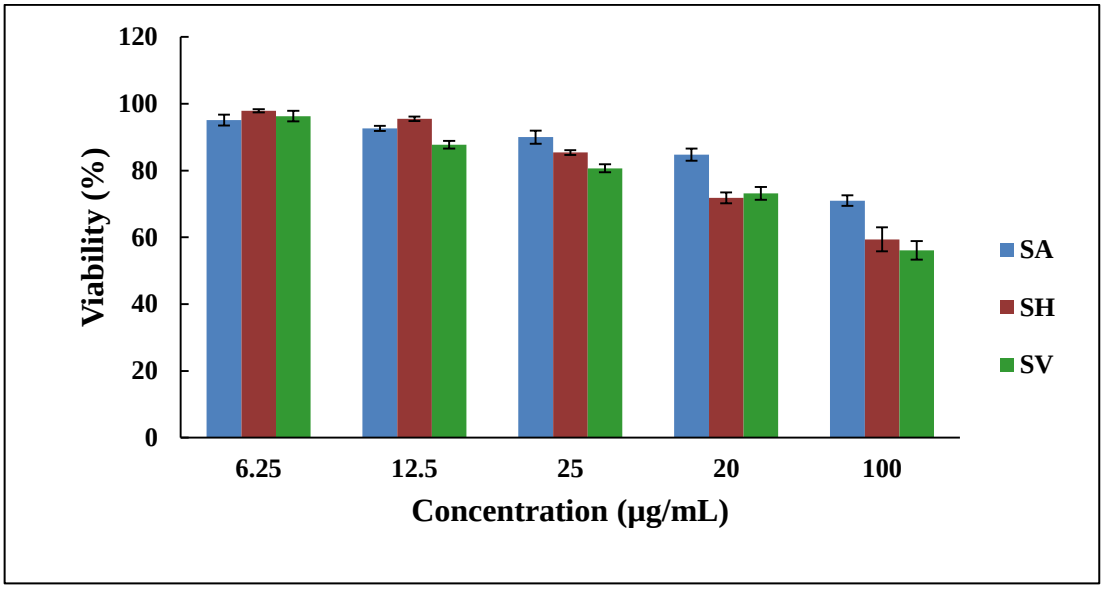


Figure 30: Evaluation of cytotoxic effect of methanolic extract of selected species of *Strobilanthes* on DLD-1 cells using MTT assay: SA - *Strobilanthes anamallaica*; SH - *Strobilanthes hamiltoniana*; SV - *Strobilanthes virendrakumarana*.

ii. *In vitro* anticancer activity using MTT assay in L929 cell line

The selected *Strobilanthes* species were examined for their cytotoxic effect on normal cell line L929 as well. The concentrations taken were 6.25, 12.5, 25. 50 and 100 µg/mL. The LC_{50} value of all the three species were high when compared with the LC_{50} in DLD1 cell line (**Table 22**). At the highest concentration (100 µg/mL) the viability percentage of *S. anamallaica*, *S. hamiltoniana* and *S. virendrakumarana* were 74.88 ± 0.97, 67.56 ± 0.42 and 71.49 ± 0.75 respectively (**Figure 31**). When scanning the viability percentage, *S. anamallaica* and *S. virendrakumarana* tried to minimise the decrease. Even though the extract of *S. hamiltoniana* had the minimum viability at its highest concentration in DLD1 cell line, their LC_{50} was not very high in L929 (normal) cell lines (**Plate 34**). The difference in LC_{50} of both *S. anamallaica* and *S. virendrakumarana* in DLD1 and L929 was high (**Plates 33, 35**). In both the cytotoxicity assays on different cell lines, *S. virendrakumarana* appeared to be a better candidate for further bioactivity studies.

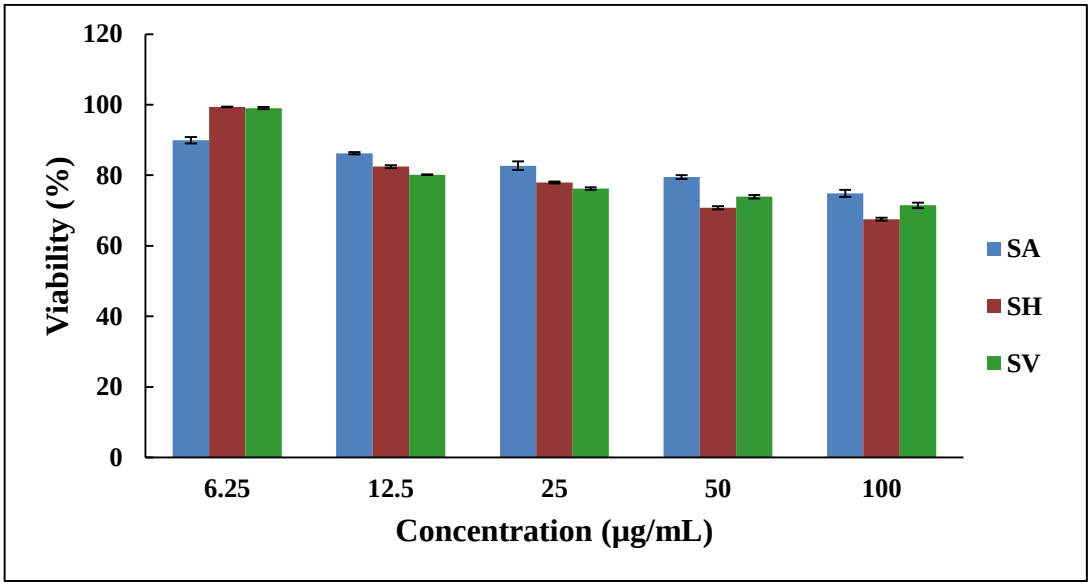


Figure 31: Evaluation of cytotoxic effect of methanolic extract of selected species of *Strobilanthes* on L929 cells using MTT assay: SA - *Strobilanthes anamallaica*; SH - *Strobilanthes hamiltoniana*; SV- *Strobilanthes virendrakumarana*.

Table 22: The effect of methanolic extracts of *Strobilanthes* species in DLD1 and L929 cell lines

Plant	LC_{50} ± SE (µg/mL)	
	DLD	L929
S. anamallaica	185.09 ± 2.89	264.03 ± 4.91
S. hamiltoniana	115.64 ± 3.46	151.21 ± 2.02
S. virendrakumarana	111.99 ± 4.91	189.79 ± 2.32

(All values are expressed in mean ± SE)

iii. Double staining using AO/EtBr

The morphological visualisation of cell death was analysed using double staining with acridine orange and ethidium bromide. With this staining, the cell death is easily distinguishable. The DLD1 cells were treated with LC_{50} concentration of each of the methanolic extracts. The double staining will give a clear-cut image of the live and dead cells. The LC_{50} concentrations of all the methanolic extracts were treated with DLD1 cell lines, and later they were stained using acridine orange – ethidium bromide

double staining technique. This method makes able to differentiate apoptotic or necrotic cells from the normal ones. Morphologically four different cells can be seen, the normal cells are having green nucleus which is clearly visible in the control. The early apoptotic ones were bright green in colour with condensed or fragmented chromatin. Whereas the late apoptotic cells have orange-stained nucleus with chromatin condensation or fragmentation. Apart from this the fourth category, that is the necrotic cells will be uniformly stained with orange colour (**Plate 36**). From the images it is clear that the cell death induced by the methanolic extract of *Strobilanthes* species is mainly due to apoptosis.

iv. Cell cycle analysis

With the help of flow cytometry, the DNA content profile and cell cycle distribution in DLD1 cells treated with LC_{50} concentration of the methanolic extracts of *S. virendrakumarana* were analysed. This extract was found to be the most potent one among the three while analysed through the antiproliferative studies using MTT assays. The population profile indicate that the apoptotic cells are clearly visible by its weaker staining intensity of propidium iodide. The cells under study were marked within a rectangle (**Figure 32**). The DNA content profile will give the cell cycle arrest stage from the cell count. The high cell counts indirectly point out the normal divisions whereas the low count represents the arrest of cell cycle in its previous stage.

The cell count in the control treatment at G0/G1, S and G2/M phase was were 72.5, 10.4 and 12.6% respectively. The cell count in extract treated cells were same at G0/G1 stage. But the count has decreased during the transition to S phase. It was 7.6%. Thus, the DNA content in G0/G1 phase of extract treated cells depict the retention of cells at that stage. The decreased DNA content indirectly distinguish the prominent apoptotic cells. The results show blocking of the transition of G0/G1 phase to S phase by the methanolic extract of *S. virendrakumarana*. The blocking was more or less similar to that

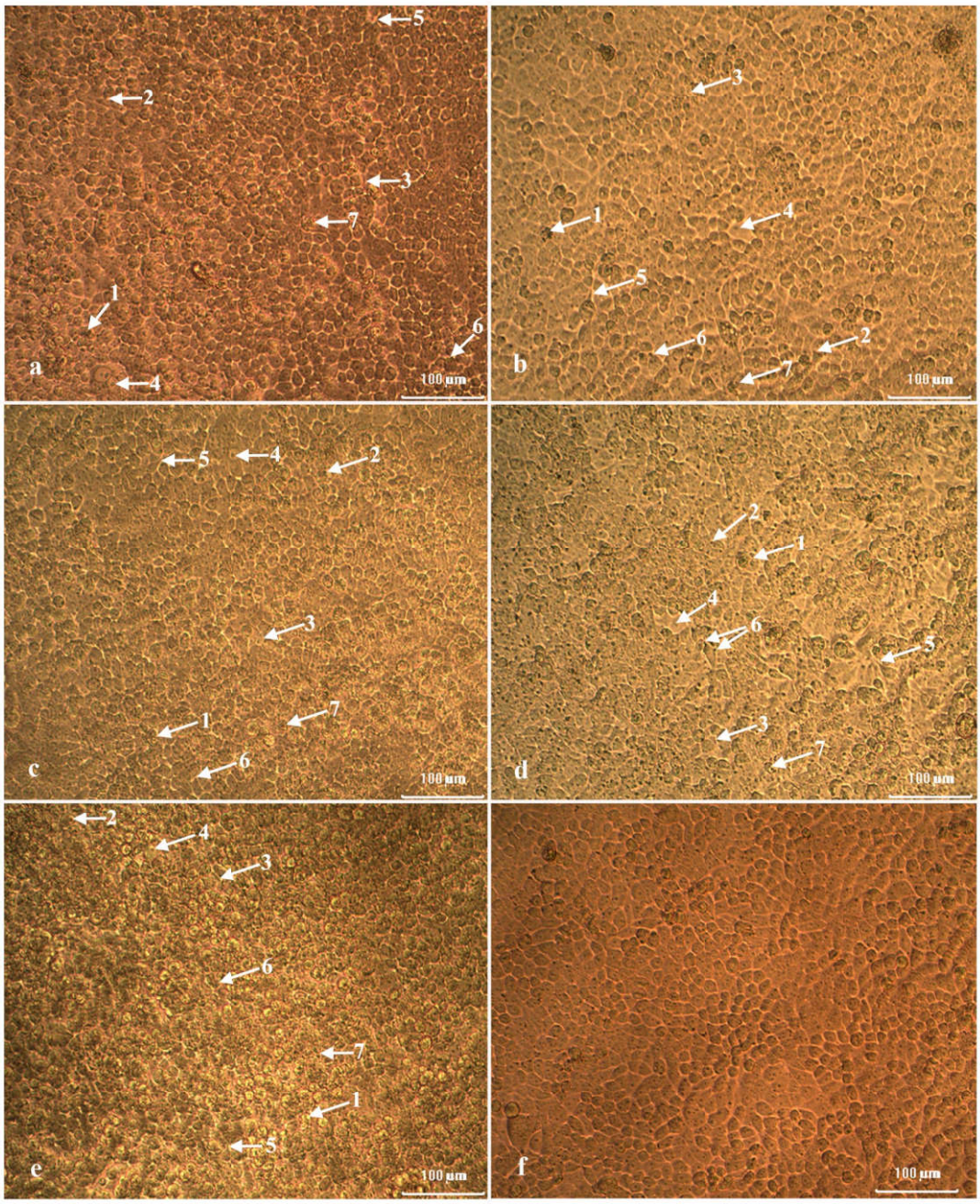


Plate 30: ***In vitro*** **cytotoxic effects of different concentrations of methanolic extracts of** ***S. anamallaica*** **on DLD1 cells, a.** 6.25 µg/mL; **b.** 12.5 µg/mL; **c.** 25 µg/mL; **d.** 50 µg/mL; **e.** 100 µg/mL; **f.** Control; **1-** apoptotic bodies; **2 -** budding; **3 -** cell shrinkage; **4 -** condensed nuclei; **5 -** echinoid spike; **6 -** membrane blebbing; **7 -** nuclear fragmentation

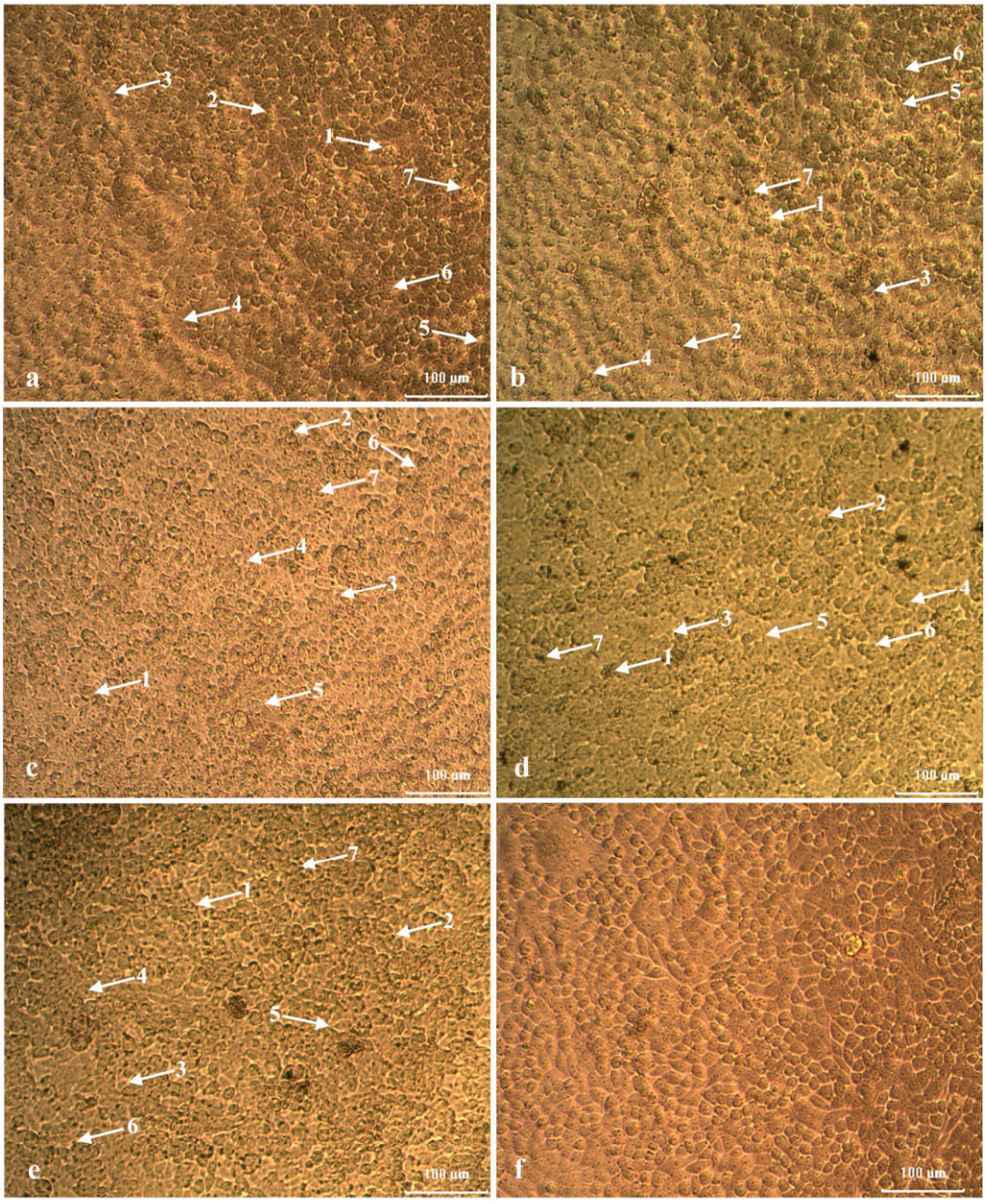


Plate 31: ***In vitro*** **cytotoxic effects of different concentrations of methanolic extracts of** ***S. hamiltoniana*** **on DLD1 cells, a.** 6.25 µg/mL; **b.** 12.5 µg/mL; **c.** 25 µg/mL; **d.** 50 µg/mL; **e.** 100 µg/mL; **f.** Control; **1-** apoptotic bodies; **2 -** budding; **3 -** cell shrinkage; **4 -** condensed nuclei; **5 -** echinoid spike; **6 -** membrane blebbing; **7 -** nuclear fragmentation

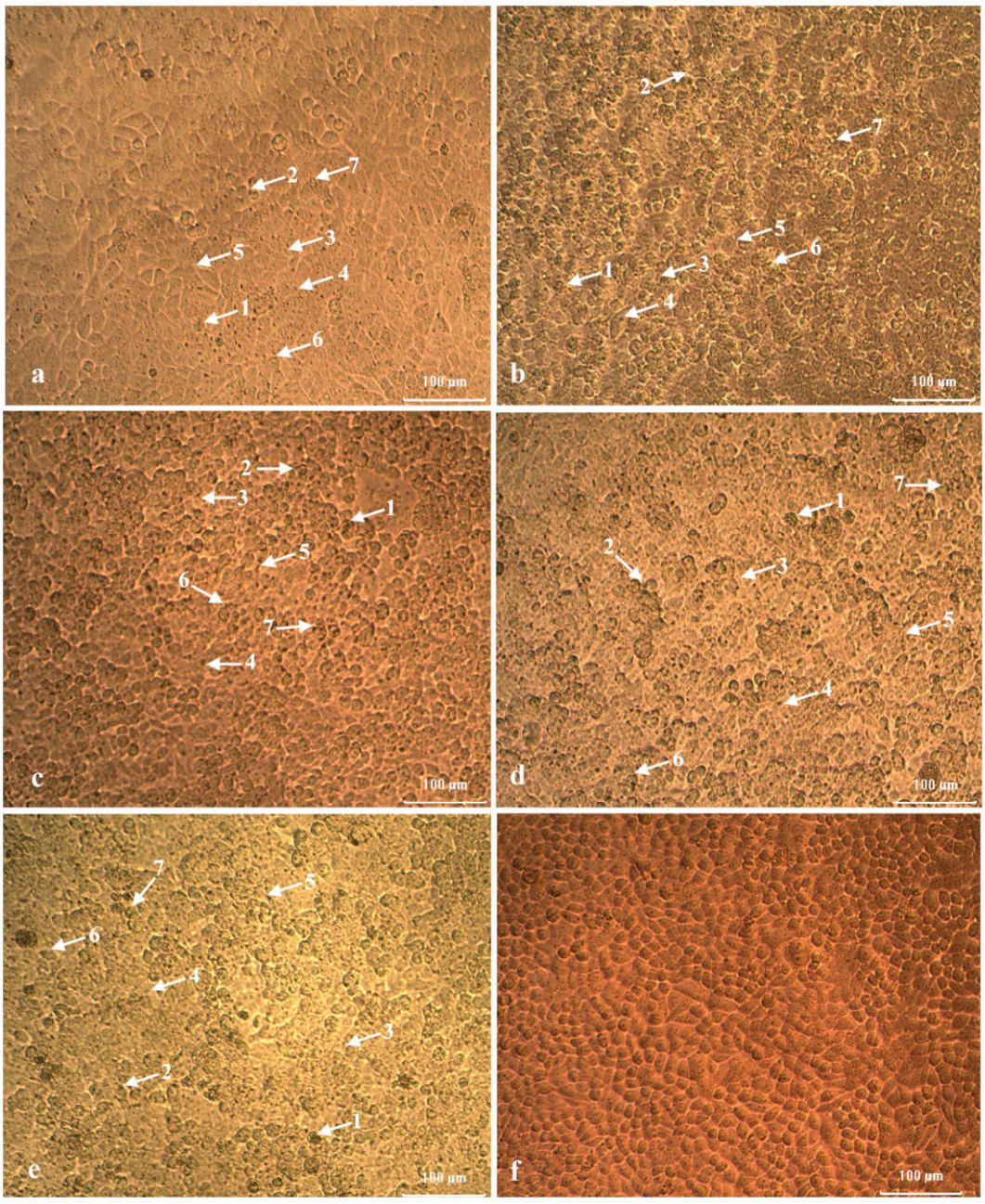


Plate 32: ***In vitro*** **cytotoxic effects of different concentrations of methanolic extracts of** ***S. virendrakumarana*** **on DLD1 cells, a.** 6.25 µg/mL; **b.** 12.5 µg/mL; **c.** 25 µg/mL; **d.** 50 µg/mL; **e.** 100 µg/mL; **f.** Control; **1-** apoptotic bodies; **2 -** budding; **3 -** cell shrinkage; **4 -** condensed nuclei; **5 -** echinoid spike; **6 -** membrane blebbing; **7 -** nuclear fragmentation

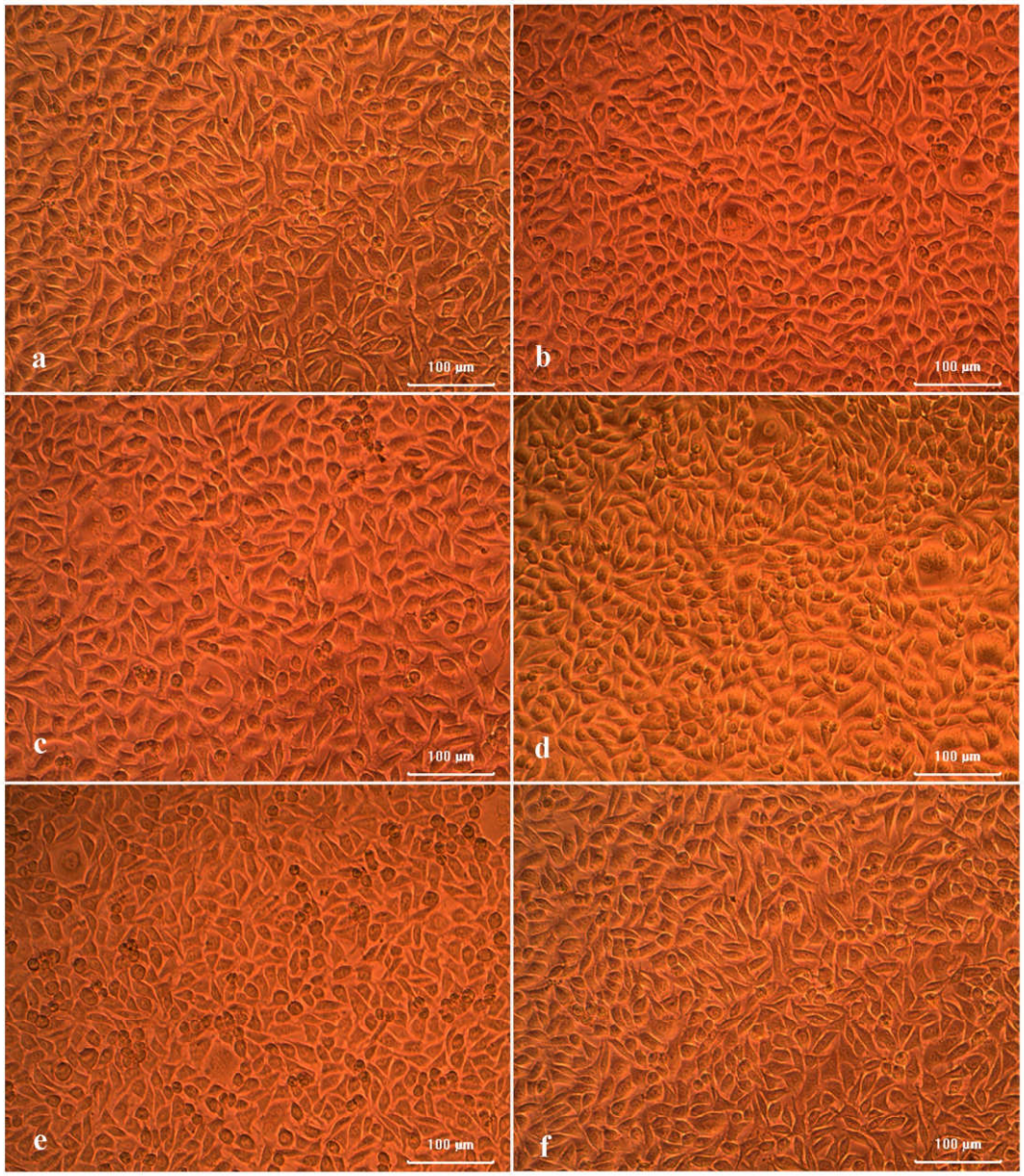


Plate 33: Effect of methanolic extract of *S. anamallaica* on L929 cell lines a. 6.25 µg/mL; **b.** 12.5 µg/mL; **c.** 25 µg/mL; **d.** 50 µg/mL; **e.** 100 µg/mL; **f.** Control

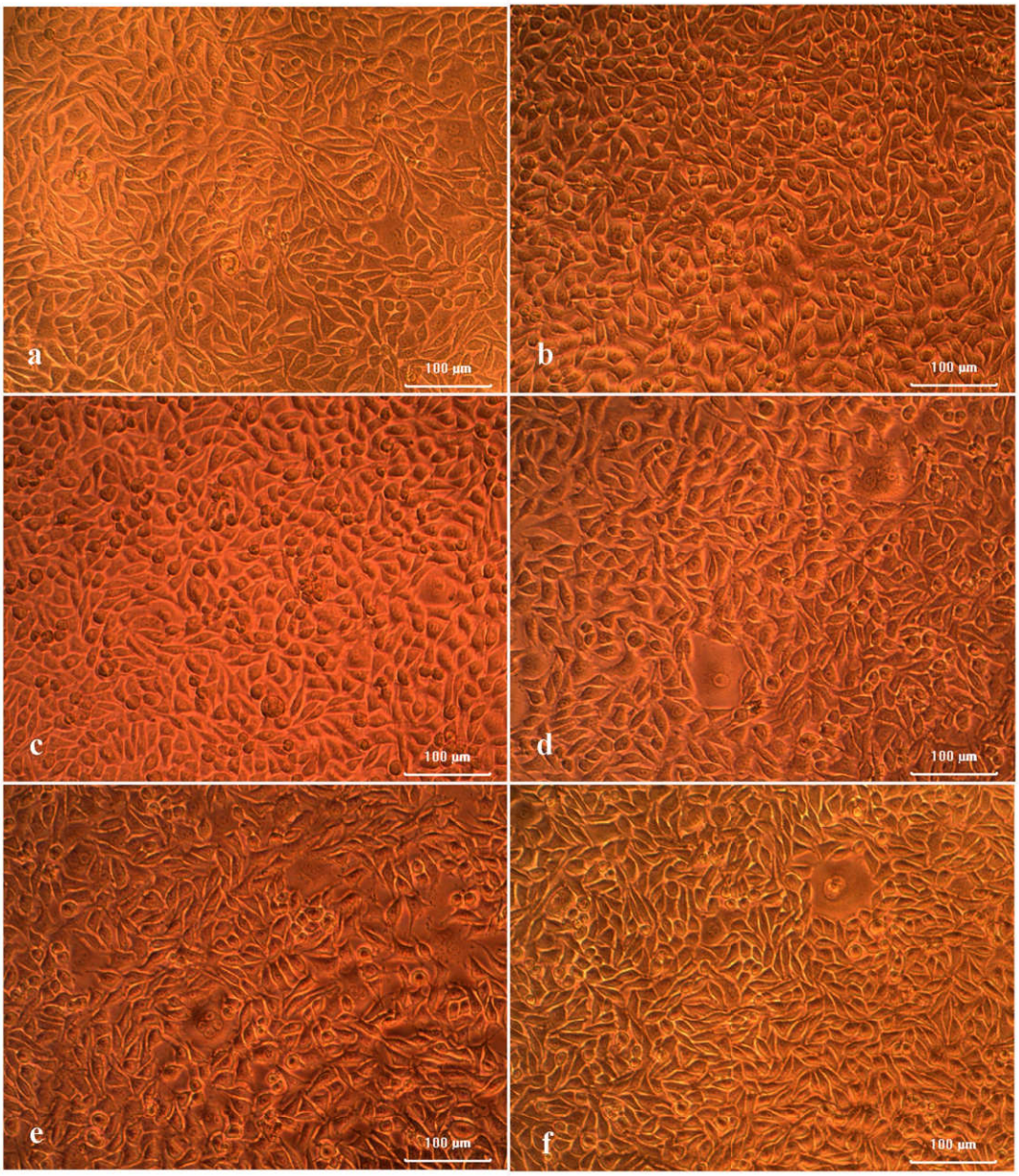


Plate 34: Effect of methanolic extract of *S. hamiltoniana* on L929 cell lines a. 6.25 µg/mL; **b.** 12.5 µg/mL; **c.** 25 µg/mL; **d.** 50 µg/mL; **e.** 100 µg/mL; **f.** Control

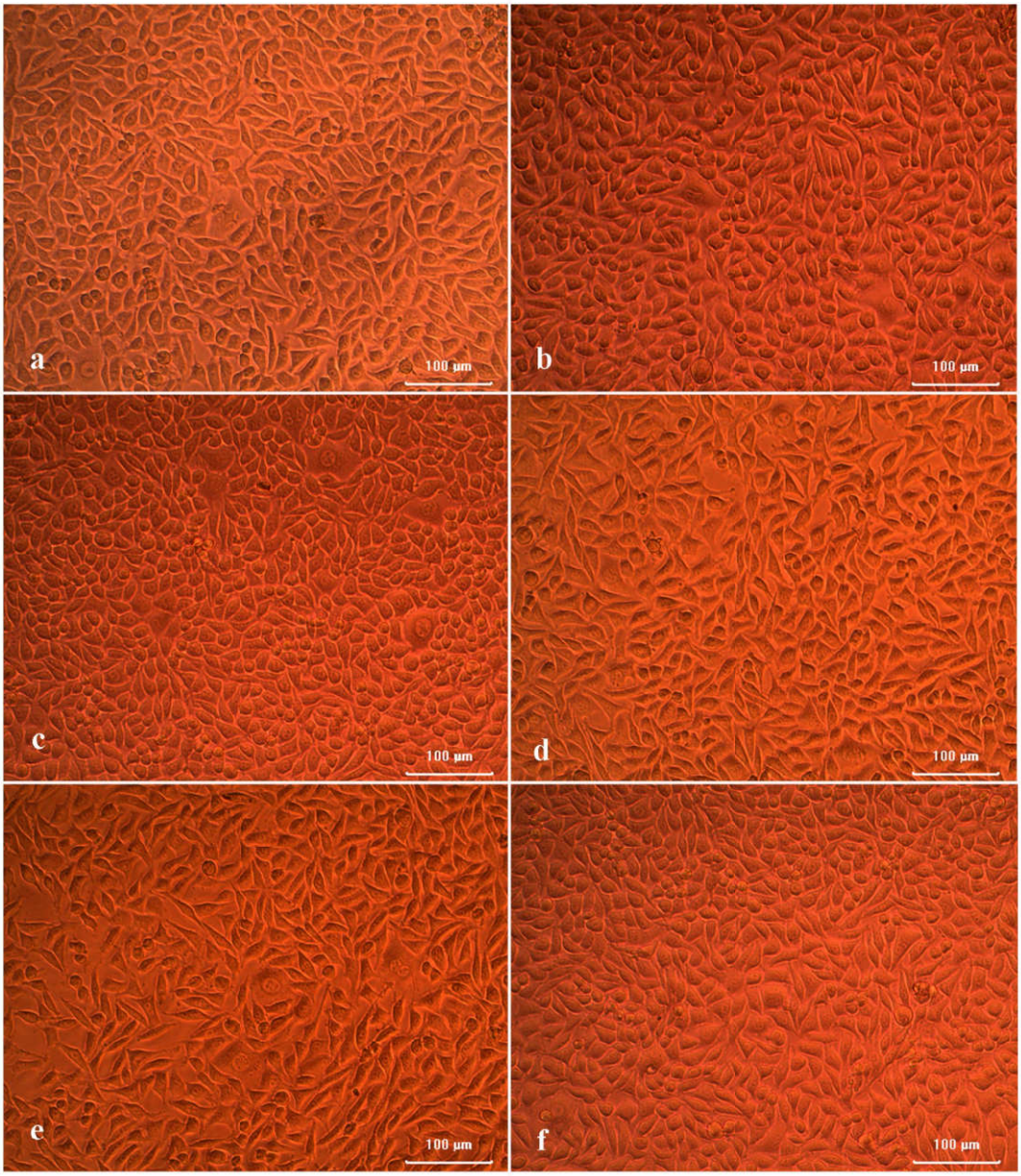


Plate 35: Effect of methanolic extract of *S. virendrakumarana* on L929 cell lines
a. 6.25 µg/mL; **b.** 12.5 µg/mL; **c.** 25 µg/mL; **d.** 50 µg/mL; **e.** 100 µg/mL; **f.** Control

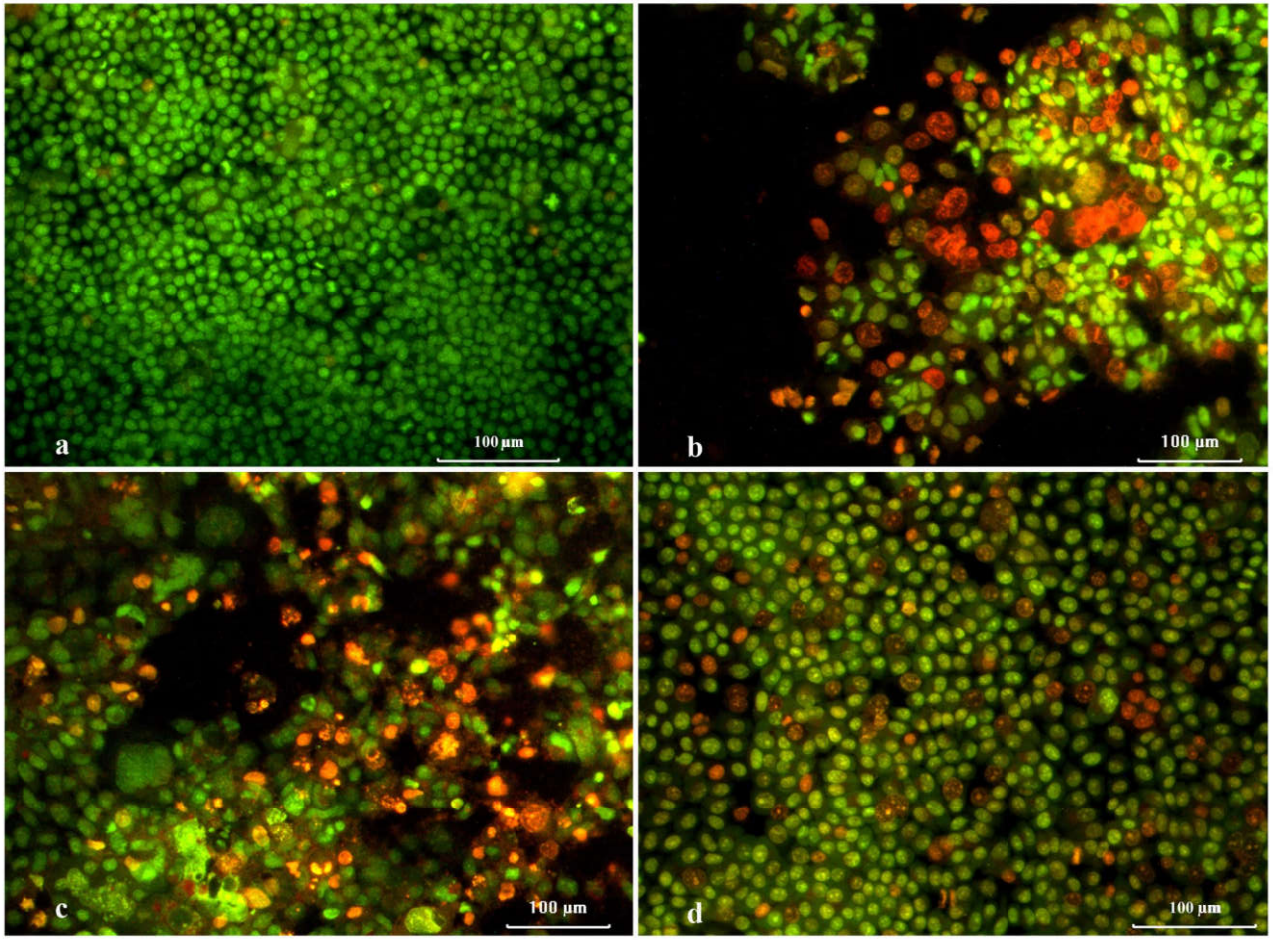


Plate 36: Detection of apoptosis by AO/EBr staining on DLD1 cells a: Control, b-d. Cells treated with methanolic extract of *Strobilanthes* species b: *S. anamallaica* (185.09 µg/mL), **c:** *S. hamiltoniana* (115.64 µg/mL), **d:** *S. virendrakumarana* (111.99 µg/mL)

of control but the percentage of cell count in G2/M phase varied tremendously. At G2/M stage the cell count was 13.5 and 10.4% for extract treatment and control respectively (**Figure 33**). The further evidence of apoptosis was indicated by the increased peak in Sub-G1/G0 phase in the extract treated cell lines.

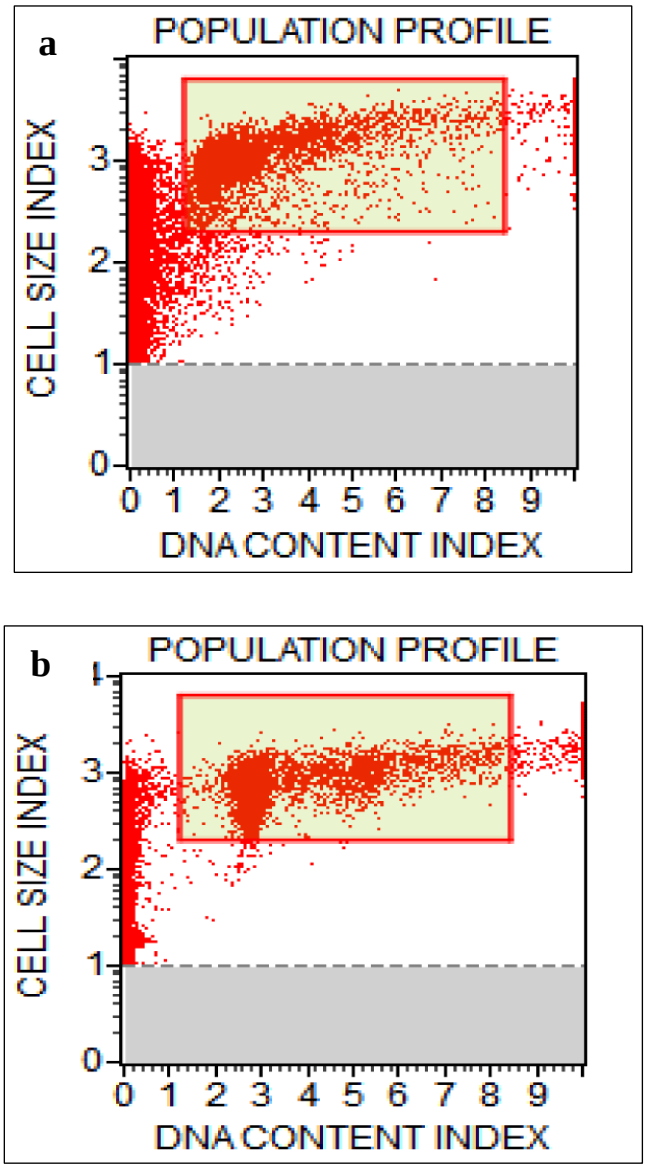


Figure 32: Population profile of DLD1 cell lines in determination of cell cycle arrest using flow cytometry: a. Control; **b.** DLD1 cells treated with methanolic extract of *S. virendrakumarana*; The rectangle represents the cells of interest excluding the cellular debris. Apoptotic cells were distinguished as shown by their weaker staining intensity of propidium iodide

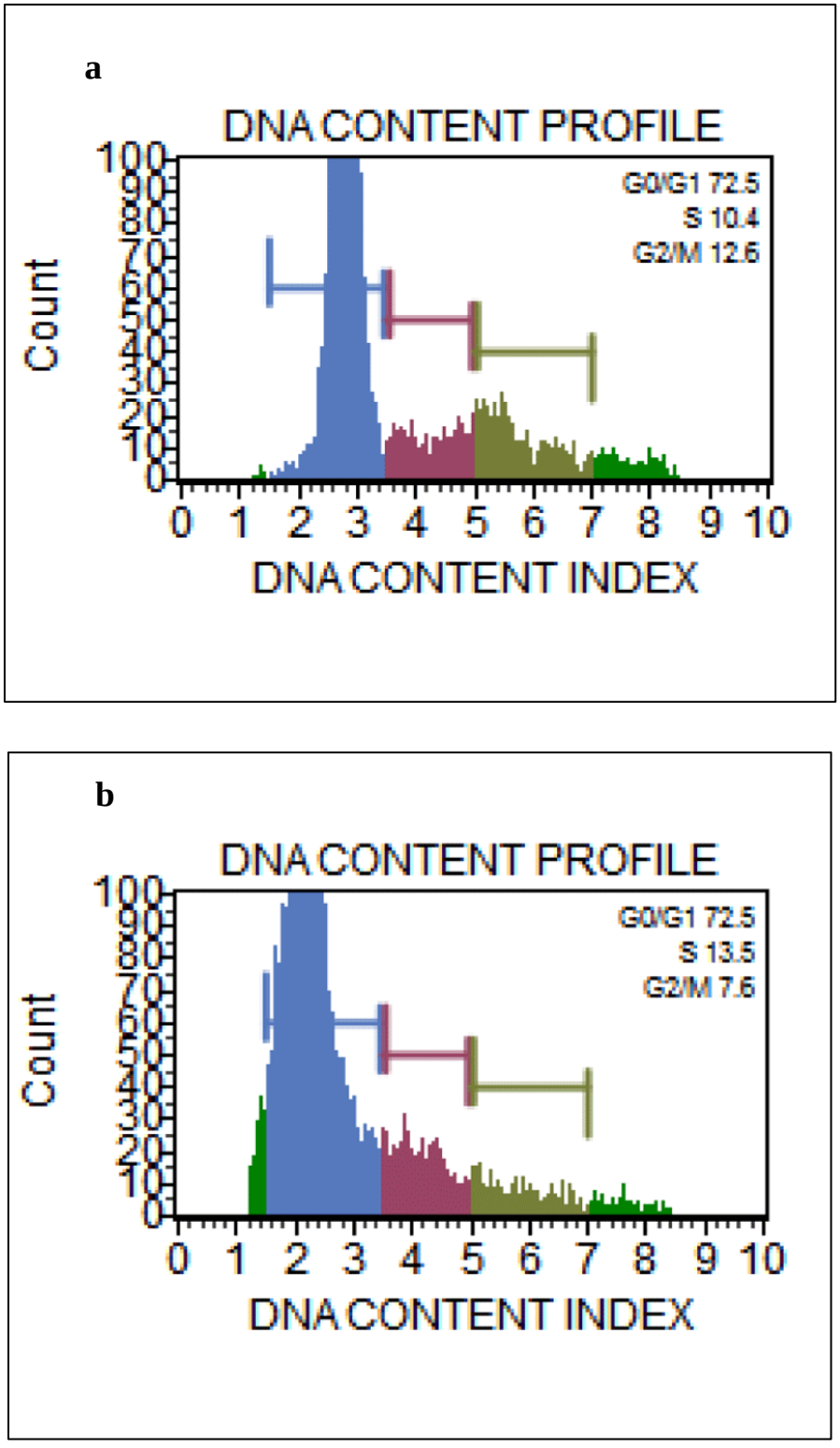


Figure 33: DNA content profile in determination of cell cycle arrest in DLD1 cell line using flow cytometry: a. Control; **b.** DLD1 cells treated with methanolic extract of *S. virendrakumarana* (24 h)

v. Gene expression studies

The effect of methanolic extract of *S. virendrakumarana* on gene expression was studied. The mechanism of antiproliferative efficacy of the extract were examined with the help of an apoptotic (Caspase 3) and an antiapoptotic gene (Bcl-2). Caspase 3 is a crucial gene involved in upregulating the apoptosis. While the Bcl-2 gene usually inhibits the cell death, there by downregulates the apoptosis. The regulation of the gene and expression change were studied by keeping GAPDH as the house keeping gene. The apoptotic efficiency of the Caspase 3 gene has enhanced by 1.88-fold when compared with the control (**Figure 34a**). That is the expression fold was almost doubled. While considering the Bcl-2 gene, the downregulation has changed 0.63-fold (**Figure 34b)**. So, the presence of the *S. virendrakumarana* extract has decreased the anti-apoptotic property of the gene. From the real time PCR method relative changes in gene expression can be analyzed using $2^{-\Delta\Delta Ct}$ method.

The change in cycle threshold (ΔC_t) value of control and sample were recorded. From this, $\Delta\Delta C_t$ values obtained for Bcl-2 was -0.63. The negative value of $\Delta\Delta C_t$ itself depicts the anti-apoptotic nature of the gene. The $\Delta\Delta C_t$ value for Caspase 3 was 1.88. The intense fluorescence in agarose gel electrophoresis indicates that the DLD1 cells treated with 111.99 µg/mL (LC_{50}) of *S. virendrakumarana* extract significantly upregulated the Caspase 3 activity. In the gel images of Bcl-2, the fluorescence was relatively lesser than the control (**Figure 35**). Hence the capacity of the methanolic extract of *S. virendrakumarana* to upregulate the apoptosis and downregulate the anti-apoptosis is clearly recorded from the gene expression analysis.

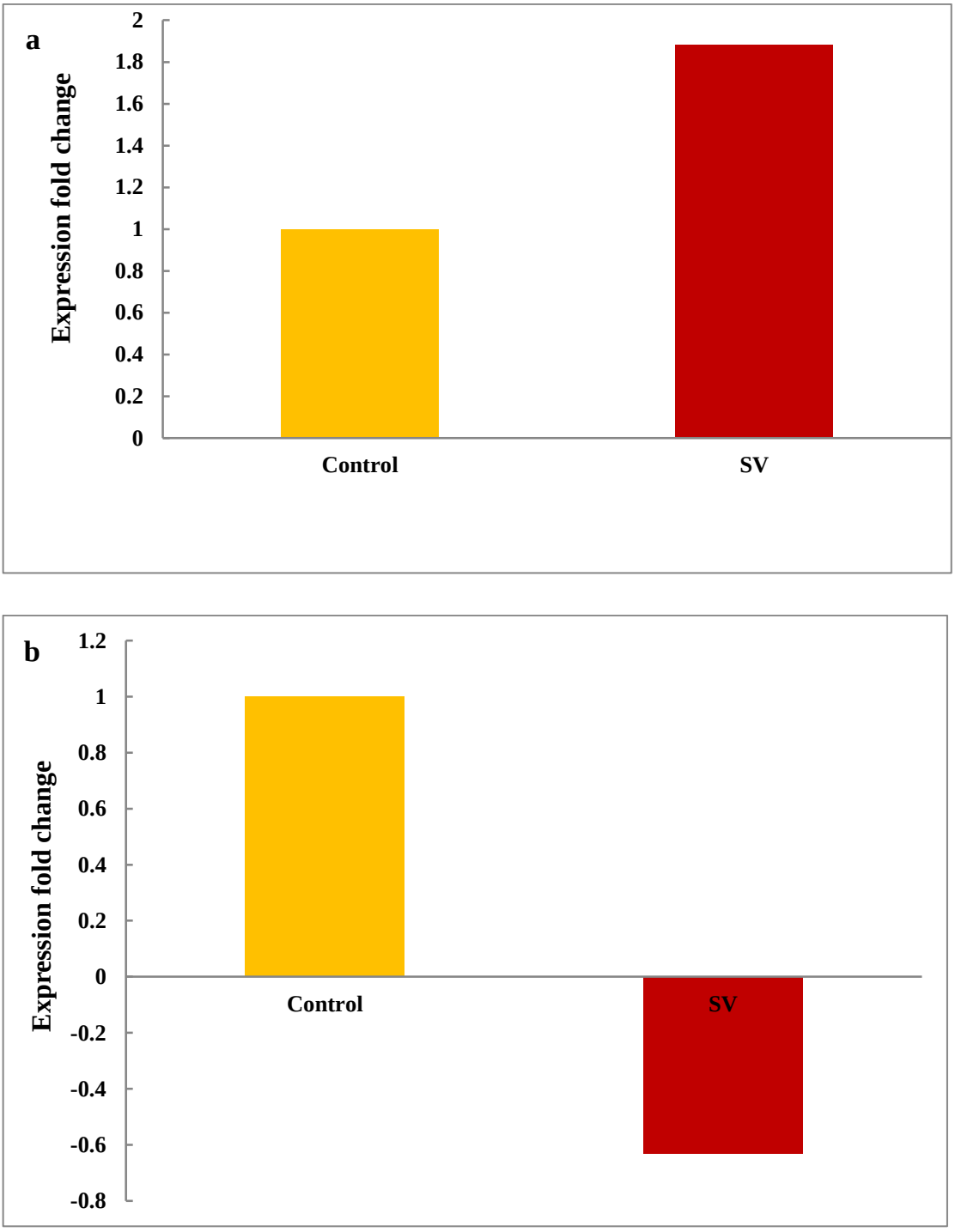


Figure 34: Expression fold changes of genes in DLD1 cells treated with shoot methanolic extract of *Strobilanthes virendrakumarana* (SV) **a.** Caspase-3 (apoptotic gene); **b.** Bcl-2 (anti-apoptotic gene)

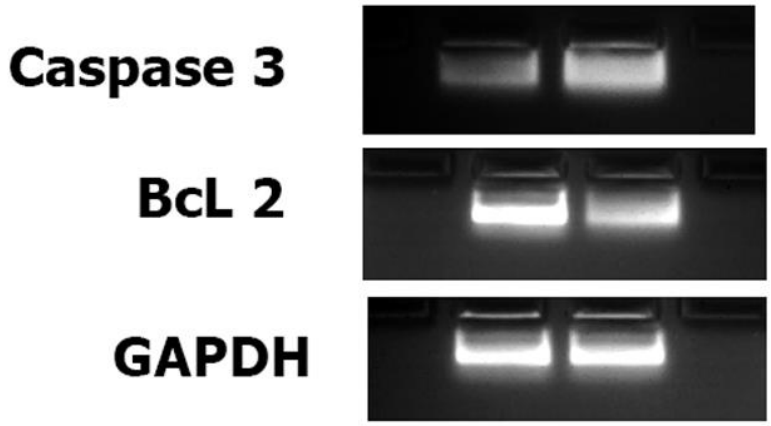


Figure 35: Expression analysis of genes Caspase-3, Bcl-2 and GAPDH using Real Time PCR

Table 5: Detailed karyomorphometric data of *S. anamallaica* somatic variant cytotype with 10 chromosomes

No.	c (µm)	s (µm)	l (µm)	R_1 (s/l)	R_2 (l/s)	I_1 (s/c%)	I_2 (l/c%)	Nature of PC
1*	2.57 ± 0.20	0.96 ± 0.08	1.60 ± 0.18	0.61 ± 0.08	1.69 ± 0.13	37.67 ± 3.30	62.33 ± 3.30	nsm(-)
2	2.17 ± 0.15	0.96 ± 0.07	1.21 ± 0.11	0.80 ± 0.06	1.26 ± 0.11	44.36 ± 1.86	55.64 ± 1.86	nm
3	1.93 ± 0.15	0.83 ± 0.09	1.12 ± 0.09	0.72 ± 0.07	1.42 ± 0.06	41.59 ± 2.34	58.40 ± 2.34	nm
4	1.51 ± 0.87	0.63 ± 0.06	0.89 ± 0.03	0.71 ± 0.05	1.44 ± 0.07	41.20 ± 1.90	58.80 ± 1.90	nm
5	1.37 ± 0.02	0.63 ± 0.02	0.75 ± 0.02	0.84 ± 0.04	1.12 ± 0.04	45.60 ± 1.30	54.30 ± 1.33	nm

(**No. -** Number of chromosome pair, **c** – total length of chromosome, **s** – short arm length, **l** – long arm length, **R_1** – arm ratio 1, **R_2** – arm ratio 2, **I_1** – centromeric index 1, **I_2** – centromeric index 2, **PC** - primary constriction, ***** - chromosome with secondary constriction, **nm** – nearly median, **nsm** – nearly submedian, values are expressed in Mean ± SE)

Table 6: Detailed karyomorphometric data of *S. anamallaica* normal cytotype with 2n = 20 chromosomes

No.	c (µm)	s (µm)	l (µm)	R_1 (s/l)	R_2 (l/s)	I_1 (s/c%)	I_2 (l/c%)	Nature of PC
1*	3.01 ± 0.07	1.14 ± 0.08	1.85 ± 0.06	0.62 ± 0.06	1.63 ± 0.15	38.24 ± 2.14	61.76 ± 2.14	nm
2*	2.32 ± 0.02	1.00 ± 0.06	1.31 ± 0.05	0.76 ± 0.07	1.33 ± 0.13	43.21 ± 2.41	56.78 ± 2.41	nm
3	1.98 ± 0.10	0.89 ± 0.04	1.09 ± 0.06	0.81 ± 0.01	1.23 ± 0.02	44.82 ± 0.39	55.18 ± 0.39	nm
4	1.84 ± 0.16	0.88 ± 0.04	1.00 ± 0.09	0.84 ± 0.03	1.19 ± 0.04	45.63 ± 0.88	54.36 ± 0.88	nm
5	1.74 ± 0.02	0.84 ± 0.06	0.92 ± 0.04	0.89 ± 0.06	1.14 ± 0.10	46.95 ± 2.23	53.05 ± 2.23	nm
6	1.62 ± 0.12	0.82 ± 0.09	0.81 ± 0.03	1.01 ± 0.19	1.06 ± 0.21	49.31 ± 4.85	50.68 ± 4.85	M
7	1.56 ± 0.09	0.74 ± 0.09	0.81 ± 0.06	0.93 ± 0.14	1.12 ± 0.15	47.64 ± 3.55	52.35 ± 3.55	nm
8	1.52 ± 0.11	0.69 ± 0.05	0.83 ± 0.06	0.83 ± 0.03	1.19 ± 0.04	45.52 ± 0.93	54.48 ± 0.93	nm
9	1.38 ± 0.11	0.63 ± 0.08	0.75 ± 0.02	0.84 ± 0.10	1.23 ± 0.15	45.35 ± 3.04	54.65 ± 3.04	nm
10	1.23 ± 0.15	0.49 ± 0.06	0.73 ± 0.10	0.68 ± 0.06	1.49 ± 0.15	40.45 ± 2.37	59.54 ± 2.37	nm

(**No.-** Number of chromosome pair, **c** – total length of chromosome, **s** – short arm length, **l** – long arm length, R_1 – arm ratio 1, R_2 – arm ratio 2, I_1 – centromeric index 1, I_2 – centromeric index 2, **PC** - primary constriction, ***** - chromosome with secondary constriction, **nm** – nearly median, **M -** median, values are expressed in Mean ± SE)

Table 7: Detailed karyomorphometric data of *S. anamallaica* somatic variant cytotype with 30 chromosomes

No.	c (µm)	s (µm)	l (µm)	R_1(s/l)	R_2 (l/s)	I_1 (s/c%)	I_2 (l/c%)	Nature of PC
1*	2.79 ± 0.15	0.90 ± 0.13	1.67 ± 0.08	0.67 ± 0.05	1.50 ± 0.12	40.00 ± 1.78	59.9 ± 1.78	nm
2*	2.22 ± 0.08	0.79 ± 0.14	1.26 ± 0.05	0.76 ± 0.03	1.32 ± 0.06	43.13 ± 1.17	56.87 ± 1.16	nm
3*	1.98 ± 0.05	0.76 ± 0.11	1.19 ± 0.10	0.67 ± 0.13	1.59 ± 0.30	39.63 ± 4.55	60.36 ± 4.56	nm
4	1.87 ± 0.09	0.67 ± 0.12	1.04 ± 0.10	0.82 ± 0.10	1.26 ± 0.18	44.07 ± 3.37	55.28 ± 3.37	nm
5	1.78 ± 0.05	0.62 ± 0.16	0.97 ± 0.02	0.84 ± 0.09	1.22 ± 0.15	45.36 ± 2.88	54.63 ± 2.88	nm
6	1.66 ± 0.05	0.92 ± 0.19	0.88 ± 0.01	0.86 ± 0.04	1.16 ± 0.05	46.40 ± 1.08	53.60 ± 1.08	nm
7	1.58 ± 0.08	0.82 ± 0.09	0.85 ± 0.09	0.89 ± 0.10	1.16 ± 0.14	46.70 ± 3.00	53.29 ± 2.95	nm
8	1.50 ± 0.070	0.60 ± 0.03	0.88 ± 0.01	0.70 ± 0.03	1.45 ± 0.06	40.9 ± 1.05	59.08 ± 1.06	nm
9	1.46 ± 0.05	0.76 ± 0.07	0.77 ± 0.05	0.90 ± 0.07	1.12 ± 0.08	47.16 ± 1.89	52.84 ± 1.87	nm
10	1.42 ± 0.04	0.68 ± 0.10	0.80 ± 0.04	0.78 ± 0.08	1.31 ± 0.14	43.48 ± 2.60	56.51 ± 2.57	nm
11	1.36 ± 0.04	0.80 ± 0.13	0.72 ± 0.03	0.89 ± 0.07	1.15 ± 0.10	46.74 ± 2.16	53.25 ± 2.16	nm
12	1.27 ± 0.05	0.72 ± 0.13	0.72 ± 0.03	0.75 ± 0.05	1.34 ± 0.78	42.86 ± 1.45	57.14 ± 1.46	nm
13	1.23 ± 0.04	0.66 ± 0.05	0.66 ± 0.04	0.86 ± 0.05	1.18 ± 0.78	46.06 ± 1.63	53.94 ± 1.63	nm
14	1.10 ± 0.01	0.65 ± 0.10	0.63 ± 0.07	0.77 ± 0.07	1.32 ± 0.12	43.36 ±2.22	56.64 ± 2.22	nm
15	0.96 ± 0.06	0.60 ± 0.07	0.59 ± 0.04	0.65 ± 0.05	1.56 ± 0.11	39.23 ± 1.77	60.76 ± 1.77	nm

(**No. -** Number of chromosome pair, **c** – total length of chromosome, **s** – short arm length, **l** – long arm length, $\mathbf{R_1}$ – arm ratio 1, $\mathbf{R_2}$ – arm ratio 2, $\mathbf{I_1}$ – centromeric index 1, $\mathbf{I_2}$ – centromeric index 2, **PC** - primary constriction, ***** - chromosome with secondary constriction, **nm** – nearly median, values are expressed in Mean ± SE)

Table 8: Summary of karyomorphometrical data of different cytotypes of *S. anamallaica*

Karyomorphometric parameters			
Feature of cytotype	Variant	Normal	Variant
Ploidy level	Monoploid	Diploid	Triploid
Somatic chromosome number	10	20	30
Karyotype formula	2 nsm (-) + 8 nm	2 M + 18 nm	30 nm
Chromosomes with secondary constriction	2	4	6
Range of chromosome length (RCL)	2.57 - 1.37 µm	2.87 - 1.23 µm	2.79 - 0.96 µm
Total chromosome length (TCL)	9.55 µm	18.19 µm	24.18 µm
Average chromosome length (ACL)	1.91 µm	1.819 µm	1.61 µm
Dispersion index (DI)	10.98	14.4	13.93
TF value (%)	41.99	45.57	45.16
Karyotype asymmetry index (As K%)	58.32	54.81	60
Syi index	72.25	83.14	79.3
Rec index	74.32	60.63	57.78
Intrachromosomal asymmetry index (A1)	0.26	0.13	0.16
Interchromosomal asymmetry index (A2)	0.25	0.08	0.29
Degree of asymmetry of karyotypes (A)	0.30	0.56	0.20

Table 9: Detailed karyomorphometric data of *S. hamiltoniana* somatic variant cytotype with 10 chromosomes

No.	c (µm)	s (µm)	l (µm)	R_1 (s/l)	R_2 (l/s)	I_1 (s/c%)	I_2 (l/c%)	Nature of PC
1*	2.80 ± 0.44	1.15 ± 0.16	1.55 ± 0.09	0.77 ± 0.04	1.40 ± 0.23	42.63 ± 4.41	57.37 ± 4.42	nm
2	2.20 ± 0.30	1.05 ± 0.16	1.16 ± 0.14	0.90 ± 0.01	1.12 ± 0.06	47.19 ± 1.28	57.81 ± 1.28	nm
3	1.83 ± 0.44	0.89 ± 0.23	0.76 ± 0.09	0.94 ± 0.09	1.07 ± 0.02	48.34 ± 0.28	51.66 ± 0.28	nm
4	1.63 ± 0.25	0.75 ± 0.14	1.03 ± 0.18	0.86 ± 0.09	1.19 ± 0.14	45.92 ± 2.70	54.08 ± 2.70	nm
5	1.42 ±0.17	0.66 ± 0.08	0.88 ± 0.13	0.87 ± 0.05	1.17 ± 0.12	46.29 ± 2.52	53.71 ± 2.52	nm

(**No. -** Number of chromosome pair, **c** – total length of chromosome, **s** – short arm length, **l** – long arm length, **R_1** – arm ratio 1, **R_2** – arm ratio 2, **I_1** – centromeric index 1, **I_2** – centromeric index 2, **PC** - primary constriction, ***** - chromosome with secondary constriction, **nm** – nearly median, values are expressed in Mean ± SE)

Table 10: Detailed karyomorphometric data of *S. hamiltoniana* normal cytotype with 2n = 20 chromosomes

No.	c (µm)	s (µm)	l (µm)	R_1 (s/l)	R_2 (l/s)	I_1 (s/c%)	I_2 (l/c%)	Nature of PC
1*	2.78 ± 0.42	1.25 ± 0.22	1.53 ± 0.20	0.81 ± 0.05	1.25 ±0.07	44.60 ± 1.45	55.39 ± 1.45	nm
2*	2.26 ± 0.37	0.99 ± 0.15	1.27 ± 0.23	0.79 ± 0.03	1.27 ± 0.05	44.00 ± 1.03	55.94 ± 1.03	nm
3	2.07 ±0.16	0.97 ± 0.11	1.20 ± 0.06	0.88 ± 0.06	1.14 ± 0.07	46.70 ± 1.68	53.26 ± 1.68	nm
4	1.87 ± 0.18	0.83 ± 0.04	1.04 ± 0.14	0.83± 0.08	1.24 ± 0.12	44.96 ± 2.58	55.04 ± 2.58	nm
5	1.77 ± 0.19	0.83 ± 0.07	0.94 ± 0.12	0.89 ± 0.05	1.12 ± 0.06	47.17 ± 1.45	52.82 ± 1.45	nm
6	1.63 ±0.20	0.67 ± 0.11	0.92 ± 0.15	0.75 ± 0.08	1.36 ± 0.14	42.61 ± 2.51	57.39 ± 2.51	nm
7	1.54 ± 0.22	0.65 ± 0.08	0.90 ± 0.13	0.76 ± 0.05	1.34 ± 0.09	42.94 ± 1.76	57.06 ± 1.76	nm
8	1.47 ± 0.22	0.66 ± 0.09	0.80 ± 0.14	0.84 ± 0.05	1.19 ±0.07	45.77 ± 1.41	54.22 ± 1.41	nm
9	1.50 ± 0.05	0.54 ± 0.08	0.76 ± 0.09	1.04 ± 0.33	1.15 ± 0.32	48.74 ± 7.56	51.26 ± 7.56	nm
10	1.18 ± 0.16	0.55 ± 0.08	0.63 ± 0.08	0.87 ± 0.03	1.15 ± 0.04	46.50 ± 0.91	53.50 ± 0.91	nm

(**No. -** Number of chromosome pair, **c** – total length of chromosome, **s** – short arm length, **l** – long arm length, $\mathbf{R_1}$ – arm ratio 1, $\mathbf{R_2}$ – arm ratio 2, $\mathbf{I_1}$ – centromeric index 1, $\mathbf{I_2}$ – centromeric index 2, **PC** - primary constriction, ***** - chromosome with secondary constriction, **nm** – nearly median, values are expressed in Mean ± SE)

Table 11: Summary of karyomorphometrical data of different cytotypes of *S. hamiltoniana*

Karyomorphometric parameters		
Feature of cytotype	Variant	Normal
Ploidy level	Monoploid	Diploid
Somatic chromosome number	10	20
Karyotype formula	10 nm	20 nm
Chromosomes with secondary constriction	2	4
Range of chromosome length (RCL)	2.8 - 1.42	2.78 - 1.18
Total chromosome length (TCL)	9.88	18.07
Average chromosome length (ACL)	1.98	1.81
Dispersion index (DI)	12.35	15.82
TF value (%)	45.54	43.94
Karyotype asymmetry index (As K%)	54.45	55.29
Syi index	84.29	79.07
Rec index	70.57	65
Intrachromosomal asymmetry index (A1)	0.14	0.21
Interchromosomal asymmetry index (A2)	0.26	0.27
Degree of asymmetry of karyotypes (A)	0.16	0.24

Table 12: Detailed karyomorphometric data of *S. virendrakumarana* somatic variant cytotype with 10+0-1B chromosomes

No.	c (µm)	s (µm)	l (µm)	R_1 (s/l)	R_2 (l/s)	I_1 (s/c%)	I_2 (l/c%)	Nature of PC
1*	2.85 ± 0.26	1.08 ± 0.1	1.78 ± 0.19	0.61 ± 0.05	1.67 ± 0.16	37.74 ± 2.25	62.26 ± 2.25	nsm (-)
2	2.43 ± 0.22	1.19 ± 0.06	1.24 ± 0.16	0.99 ± 0.05	1.03 ± 0.09	49.57 ± 2.34	50.43 ± 2.34	nm
3	2.16 ± 0.19	1.02 ± 0.08	1.14 ± 0.12	0.90 ± 0.06	1.12 ± 0.09	47.34 ± 1.84	52.66 ± 1.84	nm
4	2.06 ± 0.19	0.93 ±0.10	1.12 ± 0.11	0.84 ± 0.08	1.22 ± 0.11	45.39 ± 2.38	54.61 ± 2.38	nm
5	1.97 ±0.20	0.89 ±0.05	1.08 ± 0.15	0.84 ± 0.08	1.21 ± 0.11	45.56 ± 2.34	54.44 ± 2.34	nm
B1	0.91 ± 0.06	-	-	-	-	-	-	-

(**No. -** Number of chromosome pair, **c** – total length of chromosome, **s** – short arm length, **l** – long arm length, **R_1** – arm ratio 1, **R_2** – arm ratio 2, **I_1** – centromeric index 1, **I_2** – centromeric index 2, **PC** - primary constriction, ***** - chromosome with secondary constriction, **nm** – nearly median, **nsm** – nearly submedian, **B1** – B chromosome 1, values are expressed in Mean ± SE)

Table 13: Detailed karyomorphometric data of *S. virendrakumarana* normal cytotype 2n = 20+0-1B chromosomes

No.	c (µm)	s (µm)	l (µm)	R_1 (s/l)	R_2 (l/s)	I_1 (s/c%)	I_2 (l/c%)	Nature of PC
1*	2.95 ± 0.09	1.34 ± 0.13	1.61 ± 0.03	0.84 ± 0.09	1.22 ± 0.12	45.31 ± 2.63	54.69 ± 2.63	nm
2*	2.61 ± 0.15	1.05 ± 0.07	1.56 ± 0.08	0.67 ± 0.01	1.49 ± 0.02	40.12 ± 0.48	59.88 ± 0.48	nm
3	2.51 ± 0.06	0.89 ± 0.09	1.62 ± 0.01	0.56 ± 0.08	1.88 ± 0.31	35.47 ± 3.5	64.53 ± 3.56	nsm (-)
4	2.33 ± 0.10	1.01 ± 0.04	1.32 ± 0.12	0.78 ± 0.09	1.31 ± 0.14	43.66 ± 2.81	56.34 ± 2.81	nm
5	2.22 ± 0.14	0.96 ± 0.09	1.27 ± 0.05	0.75 ± 0.04	1.33 ± 0.07	42.96 ± 1.42	57.04 ± 1.42	nm
6	2.16 ± 0.16	1.05 ± 0.11	1.12 ±0.10	0.95 ± 0.13	1.10 ± 0.16	48.24 ± 3.53	51.76 ± 3.52	nm
7	2.04 ± 0.16	0.83 ± 0.08	1.21 ± 0.23	0.75 ± 0.17	1.55 ± 0.47	41.67 ± 6.54	58.33 ± 6.54	nm
8	1.88 ± 0.17	0.86 ± 0.08	1.03 ± 0.09	0.83 ± 0.03	1.21 ± 0.04	45.31 ± 0.84	54.69 ± .84	nm
9	1.77 ± 0.14	0.84 ± 0.06	0.93 ± 0.04	0.90 ± 0.08	1.14 ± 0.11	47.03 ± 2.37	52.97 ± 2.37	nm
10	1.62 ± 0.09	0.78 ± 0.03	0.83 ± 0.05	0.94 ± 0.02	1.06 ± 0.03	48.51 ± 0.62	51.49 ± 0.62	nm
B1	1.16 ± 0.08	-	-	-	-	-	-	-

(**No. -** Number of chromosome pair, **c** – total length of chromosome, **s** – short arm length, **l** – long arm length, **R_1** – arm ratio 1, **R_2** – arm ratio 2, **I_1** – centromeric index 1, **I_2** – centromeric index 2, **PC -** primary constriction, *** -** chromosome with secondary constriction, **nm** – nearly median, **nsm** – nearly submedian, **B1** – B chromosome 1, values are expressed in Mean ± SE)

Table 14: Detailed karyomorphometric data of *S. virendrakumarana* somatic variant cytotype with 30+0-2B chromosomes

No.	c (µm)	s (µm)	l (µm)	R_1(s/l)	R_2 (l/s)	I_1 (s/c%)	I_2 (l/c%)	Nature of PC
1*	2.87 ± 0.22	1.29 ± 0.18	1.58 ± 0.05	0.81 ± 0.09	1.27 ± 0.17	44.52 ± 3.18	55.48 ± 3.18	nm
2*	2.63 ± 0.19	1.10 ± 0.01	1.52 ± 0.19	0.75 ± 0.1	1.38 ± 0.17	42.49 ± 3.26	57.51 ± 3.25	nm
3*	2.48 ± 0.16	1.09 ± 0.1	1.38 ± 0.07	0.79 ± 0.06	1.28 ± 0.09	43.98 ± 1.87	56.02 ± 1.87	nm
4	2.23 ± 0.07	1.02 ± 0.09	1.21 ± 0.04	0.86 ± 0.09	1.20 ± 0.14	45.79 ± 2.77	54.21 ± 2.77	nm
5	2.15 ± 0.07	1.01 ± 0.04	1.15 ± 0.03	0.88 ± 0.03	1.14 ± 0.04	46.81 ± 0.76	53.19 ± 0.76	nm
6	2.06 ± 0.06	0.93 ± 0.05	1.12 ± 0.04	0.83 ± 0.06	1.21 ± 0.08	45.33 ± 1.72	54.67 ± 1.72	nm
7	1.97 ± 0.04	0.91 ± 0.04	1.06 ± 0.05	0.87 ± 0.07	1.18 ± 0.1	46.22 ± 2.15	53.78 ± 2.15	nm
8	1.90 ± 0.08	0.85 ± 0.03	1.05 ± 0.09	0.82 ± 0.09	1.25 ± 0.14	44.79 ± 2.72	55.21 ± 2.72	nm
9	1.88 ± 0.08	0.88 ± 0.06	1.00 ± 0.02	0.88 ± 0.05	1.14 ± 0.06	46.82 ± 1.32	53.18 ± 1.31	nm
10	1.82 ± 0.10	0.83 ± 0.06	0.99 ± 0.05	0.84 ± 0.02	1.18 ± 0.23	45.80 ± 0.48	54.20 ± 0.48	nm
11	1.72 ± 0.13	0.77 ± 0.13	0.96 ± 0.01	0.80 ± 0.13	1.35 ± 0.27	43.63 ± 4.57	56.37 ± 4.56	nm
12	1.69 ± 0.12	0.76 ± 0.09	0.93 ± 0.04	0.82 ± 0.08	1.25 ± 0.13	44.74 ± 2.54	55.26 ± 2.54	nm
13	1.66 ± 0.12	0.79 ± 0.87	0.87 ± 0.04	0.91 ± 0.07	1.12 ± 0.09	47.45 ± 1.96	52.55 ± 1.96	nm
14	1.62 ± 0.14	0.73 ± 0.08	0.88 ± 0.07	0.83 ± 0.04	1.21 ± 0.06	45.19 ± 1.21	54.81 ± 1.21	nm
15	1.51 ± 0.13	0.69 ± 0.06	0.82 ± 0.09	0.86 ± 0.05	1.17 ± 0.07	46.12 ± 1.45	53.88 ± 1.45	nm
B1	0.86 ± 0.14	-	-	-	-	-	-	-
B2	0.77 ± 0.10	-	-	-	-	-	-	-

(**No. -** Number of chromosome pair, **c** – total length of chromosome, **s** – short arm length, **l** – long arm length, $\mathbf{R_1}$ – arm ratio 1, $\mathbf{R_2}$ – arm ratio 2, $\mathbf{I_1}$ – centromeric index 1, $\mathbf{I_2}$ – centromeric index 2, **PC** - primary constriction, ***** - chromosome with secondary constriction, **nm** – nearly median, **B1** – B chromosome 1, **B2** – B chromosome 2, values are expressed in Mean ± SE)

Table 15: Detailed karyomorphometric data of *S. virendrakumarana* somatic variant cytotype with 40+0-2B chromosomes

No.	c (µm)	s (µm)	l (µm)	R_1(s/l)	R_2 (l/s)	I_1 (s/c%)	I_2 (l/c%)	Nature of PC
1*	2.74 ± 0.24	1.23 ± 0.19	1.51 ± 0.07	0.81 ± 0.11	1.29 ± 0.19	44.21 ± 3.52	55.79 ± 3.52	nm
2*	2.51 ± 0.23	1.10 ± 0.16	1.41 ± 0.12	0.79 ± 0.12	1.32 ± 0.17	43.52 ± 3.46	56.48 ± 3.46	nm
3*	2.29 ± 0.30	0.91 ± 0.13	1.39 ± 0.18	0.65 ± 0.02	1.54 ± 0.04	39.39 ± 0.68	60.61 ± 0.68	nm
4	2.28 ± 0.33	1.08 ± 0.15	1.21 ± 0.21	0.82 ± 0.08	1.24 ± 0.12	44.87 ± 2.48	55.13 ± 2.48	nm
5	2.27 ± 0.37	0.99 ± 0.13	1.27 ± 0.24	0.80 ± 0.06	1.26 ± 0.08	44.45 ± 1.75	55.55 ± 1.75	nm
6	1.98 ± 0.24	0.90 ± 0.11	1.08 ± 0.15	0.86 ± 0.09	1.20 ± 0.14	45.85 ± 2.72	54.15 ± 2.72	nm
7	1.90 ± 0.19	0.86 ± 0.08	1.05 ± 0.13	0.83 ± 0.07	1.22 ± 0.10	45.27 ± 2.13	54.73 ± 2.13	nm
8	1.86 ± 0.19	0.88 ± 0.09	0.99 ± 0.10	0.89 ± 0.03	1.13 ± 0.04	47.05 ± 0.81	52.95 ± 0.81	nm
9	1.76 ± 0.14	0.74 ± 0.06	1.03 ± 0.09	0.73 ± 0.04	1.39 ± 0.09	41.89 ± 1.61	58.11 ± 1.61	nm
10	1.71 ± 0.14	0.80 ± 0.07	0.90 ± 0.09	0.90 ± 0.12	1.15 ± 0.16	47.03 ± 3.40	52.97 ± 3.40	nm
11	1.66 ± 0.13	0.79 ± 0.07	0.87 ± 0.06	0.90 ± 0.04	1.12 ± 0.05	47.18 ± 1.10	52.82 ± 1.10	nm
12	1.61 ± 0.13	0.69 ± 0.08	0.91 ± 0.07	0.77 ± 0.11	1.33 ± 0.13	43.12 ± 2.54	56.88 ± 2.54	nm
13	1.56 ± 0.17	0.73 ± 0.09	0.83 ± 0.09	0.89 ± 0.09	1.15 ± 0.12	46.85 ± 2.59	53.15 ± 2.59	nm
14	1.58 ± 0.13	0.72 ± 0.06	0.86 ± 0.07	0.84 ± 0.06	1.20 ± 0.08	45.53 ± 1.73	54.47 ± 1.73	nm
15	1.49 ± 0.09	0.70 ± 0.05	0.79 ± 0.05	0.90 ± 0.05	1.12 ± 0.06	47.31 ± 1.30	52.69 ± 1.30	nm
16	1.51 ± 0.10	0.75 ± 0.09	0.76 ± 0.09	1.05 ± 0.20	1.03 ± 0.19	50.11 ± 4.75	49.89 ± 4.75	M
17	1.51 ± 0.09	0.72 ± 0.07	0.78 ± 0.10	1.11 ± 0.22	0.96 ± 0.16	51.74 ± 4.54	48.26 ± 4.54	M
18	1.48 ± 0.11	0.65 ± 0.06	0.83 ± 0.08	0.79 ± 0.08	1.30 ± 0.13	43.83 ± 2.46	56.17 ± 2.46	nm
19	1.43 ± 0.11	0.69 ± 0.04	0.75 ± 0.07	0.92 ± 0.03	1.10 ± 0.14	47.75 ± 0.90	52.25 ± 0.90	nm
20	1.34 ± 0.09	0.67 ± 0.04	0.67 ± 0.06	1.00 ± 0.04	1.00 ± 0.03	50.01 ± 0.85	49.99 ± 0.86	M
B1	0.82 ± 0.05	-	-	-	-	-	-	-
B2	0.80 ± 0.05	-	-	-	-	-	-	-

(**No. -** Number of chromosome pair, **c** – total length of chromosome, **s** – short arm length, **l** – long arm length, **R_1** – arm ratio 1, **R_2** – arm ratio 2, **I_1** – centromeric index 1, **I_2** – centromeric index 2, **PC** - primary constriction, ***** - chromosome with secondary constriction, **nm** – nearly median, M – median, **B1** – B chromosome 1, **B2** – B chromosome 2, values are expressed in Mean ± SE)

Table 16: Summary of karyomorphometrical data of different cytotypes of *S. virendrakumarana*

Karyomorphometric parameters				
Feature of cytotype	Variant	Normal	Variant	Variant
Ploidy level	Monoploid	Diploid	Triploid	Tetraploid
Somatic chromosome number	10+0-1B	20+0-1B	30+0-2B	40+0-2B
Karyotype formula	2 nsm (-) + 8 nm + 0-1 fragment	2 nsm (-) + 18 nm + 0-1 fragment	30 nm + + 0-2 fragment	6 M + 34 nm + + 0-2 fragment
Chromosomes with secondary constriction	2	4	6	8
Range of chromosome length (RCL)	2.85 – 1.95 µm	2.95 – 1.62 µm	2.87 – 1.51 µm	2.74 – 1.34 µm
Total chromosome length (TCL)	23.85 µm	45.34 µm	62.01 µm	74.56 µm
Average chromosome length (ACL)	2.29 µm	2.21 µm	2.01 µm	1.82 µm
Dispersion index (DI)	7.31	8.48	8.76	10.24
TF value (%)	44.6	43.5	50.3	45.5
Karyotype asymmetry index (As K%)	55.4	56.6	49.7	54.5
Syi index	80.35	76.88	82.63	83.46
Rec index	80.49	74.88	70.13	66.55
Intrachromosomal asymmetry index (A1)	0.18	0.21	0.17	0.15
Interchromosomal asymmetry index (A2)	0.15	0.18	0.20	0.22
Degree of asymmetry of karyotypes (A)	0.20	0.24	0.18	0.16

(nm – nearly median, nsm – nearly submedian, M – median)

DISCUSSION

PHASE I - CYTOGENETICAL STUDIES

The cytological identity of a species is elucidated from karyomorphological data. Karyomorphology tends to focus on the evolutionary status shared by the species. Though chromosome numbers in the genus *Strobilanthes* were reported earlier, the detailed information regarding the karyomorphological parameters are few. Chromosomes are useful materials in cytogenetics as genetic information is passing through them. The interpretation of karyotype evolution among species requires not only the chromosome number but also its karyomorphology. The insufficient data of ecology and morphology of species in explaining ancestry can be rectified by the cytotaxonomic and molecular pieces of evidence.

The changes in the number and structure of chromosomes can be linked to the evolution and speciation of plants (Astuti et al., 2017). Karyotyping mainly deals with the number, size and position of centromere which are important in finalising the evolutionary descent of a species. The earlier concept by Stebbins and Levitsky has purely relied upon inter-chromosomal and intra-chromosomal asymmetry. The first one is the diversity of chromosome length within a set whereas, the latter depends on the position of the centromere. The recent concepts add an additional framework for suggesting symmetry. The presence of median chromosomes signify primitiveness and terminal centromeres represent advanced ones (Imai et al., 2001). All the diploid cytotypes under investigation point out the primitive character due to their nearly median chromosomes with a few median and nearly sub-median ones (**Plates 5, 6, 11, 12, 15, 16**). According to Abraham and Prasad (1983), the different classes of chromosomes have terminal (T), median (M), submedian (SM), subterminal (ST), nearly terminal

(nt), nearly median (nm), nearly submedian (nsm+ and nsm-) and nearly subterminal (nst+ and nst-) centromeres.

Karyotypic analysis has been successfully employed to estimate the intraspecific and interspecific levels of taxonomic relationships between species (Beevy & Bai, 2013). With the help of due standardisation, the basic chromosome number in metaphase plates of selected *Strobilanthes* species were found to be x = 10 with a mixoploid karyotype (**Plates 3, 4, 9, 10, 13, 14**). The previous reports suggest various basic chromosome numbers in *Strobilanthes* (**Table 1**).

Even though many cytological works have been reported in Acanthaceae, only very few reports on South Indian species of *Strobilanthes* are available. The present study report the mixoploidy in the genus *Strobilanthes* for the first time. Mixoploidy or polysomaty is the occurrence of diploid and polyploid cells together (Zhou et al., 2011) in which diploid chromosome number dominates over other polyploid cells (Yudanova, 2003). The dominating cell fraction under study was 2n = 20 with a small fraction of other ploidy levels. Hence, the somatic chromosome number was determined to be 2n = 2x = 20.

The basic chromosome number is important in determining the systematic position of a taxon. In these species, both hypoploid and hyperploid cells along with the normal diploid complement were present except for *S. hamiltoniana,* in which only two ploidy (10, 20) levels were seen (**Tables 9, 10 & Plates 9-12**). *S. anamallaica* recorded three cytotypes (**Plates 3-8**). The karyotype formulae for the variant cytotypes were 2 nsm (-) + 8 nm and 30 nm respectively for monoploid and triploid cells (**Tables 5, 6, 7**). The chromosome counts of these three species are revealed for the first time. Hence it adds additional data to the depository of the chromosome

database which can be further used for determining the evolutionary scenario of these species.

Structural variations in plant genomes can be due to environmental stress which ultimately leads to mixoploidy, aneuploidy and chromosome rearrangements. In flowering plants, mixoploidy is maintained by vegetative and apomictic propagation (Amosova et al., 2015). According to them, mixoploidy is a cytogenetic abnormality common in stress-afflicted plants. The presence of more than one chromosome number in a cellular population is defined as mixoploidy (King et al. 2006). Mixoploidy, meiotic abnormalities and polyploidy had induced the production of unbalanced gametes, thereby decreasing the pollen viability and seed production in *Lippia alba* (Pierre et al., 2011). In plants, where natural hybridization is common, mixoploidy may be a consequence of the hybridization between closely related species. Generally, triploid plants arise by the hybridization of tetraploids and diploids. Hence most triploid cytotypes occur in the mixoploid populations containing diploids and tetraploids (Ao, 2008).

When plants are exposed to abiotic stresses, karyotypes tend to evolve polyploidy which is suitable for that environment (Mayrose et al., 2010). It is believed that polysomaty arises from diploid meristems at a particular stage of ontogenesis. It is most common in plants with vegetative and apomictic propagation (Kunakh, 1995). The instability in the cell genome within a meristem can induce the ability for hybridisation and polyploidisation. These in turn can lead to epigenetic and epigenomic rearrangements (Kunakh et al., 2008).

The karyotype formulae for the diploid somatic cells of *S. anamallaica, S. hamiltoniana* and *S. virendrakumarana* were 18 nm + 2 M, 20 nm and 2 nsm (-) + 18 nm respectively (**Tables 8, 11, 16**). There are no previous reports on the karyotype formulae of these species. Govindarajan &

Subramanian had given the range of chromosome length in the genus *Strobilanthes* as 4.5-0.8 µm from the study of ten species. The karyotypes of all the ten species revealed short and very short chromosomes in its normal chromosome complement except for *S. pulneyensis* and *S. lawsoni*. These two species have medium-sized chromosomes too. The centromere position in the genus was mainly median to sub-median with a few having sub terminal ones (Govindarajan & Subramanian 1983, 1985). In accordance with their results, the chromosome length range in the present study was found to be 2.87 - 1.23, 2.78 - 1.18 and 2.95 - 1.62 µm for the diploid chromosome complement of *S. anamallaica, S. hamiltoniana* and *S. virendrakumarana* respectively.

The gradual reduction in chromosome size was shown in all the ploidy levels of the three species. The total chromosome length (TCL) in all the higher ploidies is not the sequential increase of the lower ploidy. TCL in somatic variant cytotype with 10 chromosomes of *S. anamallaica, S. hamiltoniana* and *S. virendrakumarana* was found to be 9.55, 9.88 and 23.85 µm respectively. However, their corresponding TCL in 2n = 20 was 18.19, 18.07 and 45.34 µm respectively (**Tables 8, 11, 16**). That is the total chromosome length of 2n = 20 in the diploid cytotype do not exhibit the exact doubling of the monoploid. The same pattern was observed in higher ploidies also. This type of duplication does not accompany a proportionate increase in the length of the karyotype, so these do not fall under true polyploidy (Tena-Flores et al., 2014). The chromosomes significantly compact during interphase, however upon entering into mitosis, chromatin condenses further and form individual chromosomes which are captured by the mitotic spindle apparatus (Antonin & Neumann, 2016). A similar condensation index may be responsible for the similarity in chromosome size and gross morphology among the selected species (Biswas & Mukhopadhyay, 2020).

For understanding the basic structure of the genetic complement, somatic chromosome number determination and ploidy level estimation is important (Chakraborti et al. 2010). Longer chromosomes, having median centromeres with equal length of chromosome arms and low basic chromosome numbers are features of symmetrical karyotypes. According to Stebbins (1971) in relation to phylogeny and evolutionary processes, asymmetrical karyotypes are more advanced than symmetrical ones. More advanced species will have asymmetrical karyotypes. When a karyotype has predominant chromosomes with terminal or subterminal centromeres and highly heterogenous chromosome sizes, it is an asymmetric karyotype (Uysal et al., 2015). In contradicting to that, the prevalence of median and nearly median chromosomes dominate in this genus results in the highly symmetrical karyotype (Lekhak et al., 2018). Symmetry does not depend upon chromosome number or chromosome size (Tabur et al., 2012).

S. virendrakumarana have B-chromosomes in its chromosome complement. The karyotype formulae of the somatic variant cytotypes *viz.*, 10+0-1B, 30+0-2B and 40+0-2B were 2 nsm (-) + 8 nm + 0-1 fragment, 30 nm + 0-2 fragments and 6 M + 34 nm + 0-2 fragments respectively (**Tables 12, 13, 14, 15 & Plates 13-20**). Generally, Bs are said to be formed of heterochromatin, but this is not the same in all cases. In *Scilla hohenackeri*, Bs are with less heterochromatin than normal chromosomes (Greilhuber & Speta, 1976). B-chromosomes (Bs) are supernumerary or mini chromosomes originated from autosomal complements and they perpetuate in an organism by their species-specific evolutionary pathway (Kumar & Singh, 2021). It is discovered in fungi, birds, fishes, plants and animals. They are one among the genetic elements which do not obey the Mendelian law of inheritance, hence they act as a parasitic element with a high transmission rate of more than 0.5 (Bednářová et al. ,2021). Bs are the by-products of karyotype evolution. Stornioli et al. (2021) described the behavior of Bs as ‘selfish’ due to their

autonomous inheritance to ensure their survival in populations. It does not influence phenotypic character and hence the only way to find them are the cytological preparations.

Regarding the existence of Bs, several researchers have put forward many suggestions such as they are restricted to diploids or tetraploids or completely absent in tetraploids (Dwivedi & Kumar, 2019). In diploid (2n = 20+0-1B) and hypoploid cell (10+0-1B) only one B chromosome was found whereas in hyperploid cells, an increase in the number of Bs were observed (**Plates 13, 14, 15, 16**). In reference to the reports by Haga (1961) when the ploidy level increases, the number of Bs also increases. Likewise in *S. virendrakumarana,* hyperploid cells were found to have 2 Bs (**Plates 17, 18, 19, 20**) and hypoploid and diploid cells have a single B chromosome. The disturbance of Bs in mitosis and meiosis may be a reason for its occurrence as one or two (Boroń et al., 2020). Bs never duplicates or combines with any other chromosomes and due to their non-mendelian inheritance, they are not visible in all sister cells of root meristem under consideration. The repeated non-coding DNA of heterochromatic Bs influences their maintenance in mitotic cells as well as meiotic cells in gametes (Ramos et al., 2017).

The previous report in *Salvia* species suggests its oil poor nature due to decreased metabolic activities influenced by the presence of Bs (Mani & Thoppil, 2005). Thus, Bs may have a direct or indirect influence on phytochemical characters. The seedlings containing B-chromosomes have a narrow competitive advantage over others during germination under high mortality circumstances (Holmes & Bougourd, 1989). Hence selection plays a significant role in B polymorphism and its maintenance.

The presence of Bs influence the cell cycle and may permit the formation of more chiasmata (Camacho, 2005). The 'odd-even effect' suggests that the chiasma frequency of A-chromosomes is enhanced by the

presence of mini-chromosomes (Bs) with an odd number rather than even (Ghaffari & Bidmeshkipoor, 2002). Morphologically B-carrier plants cannot be distinguished from non-carrier plants. Even the carrier plants show high chiasma frequency and the fertility is slightly reduced with the occurrence of Bs. Hence these chromosomes can play a role in generating new allelic combinations and genome evolutionary processes (Kumar & Singh, 2021). Green (1990) reported the ribosomal DNA cistron of Bs mostly in the form of nucleolar organizer regions, which gives an advantage to them during meiosis. There are genes on Bs which control the non-disjunction in maize and rye (Beukeboom, 1994) and thereby affecting the normal cell division. Bs may also increase the competition with normal chromosomes for replication machinery within the cell for certain enzymes. So, it can be inferred that polyploids can tolerate Bs than normal diploids since they have a larger number of enzymes. The presence of one to two B-chromosomes in different ploidy levels of chromosome constitution may improve the tolerance of the plant. The establishment of polyploidy is mainly due to vegetative reproduction and perennial habitat (Soltis & Soltis., 2000).

The unusual chromosome numbers in plants can be due to various reasons. In Acanthaceae, it is mainly due to polyploidy and aneuploidy (Daniel et al., 1990). The present study reports mixoploidy in *Strobilanthes* for the first time. Mixoploidy or polysomaty is due to natural, artificial and chemical factors which are documented in many species that are resorted to vegetative means of propagation. The major report of mixoploidy was in anther culture (Xin-Hua et al., 2010). Their presence is mostly in genera having small-sized chromosomes (Truta et al., 2011). It is observed in extreme conditions such as near borders of or on physiological limits of the species existence with some potential adaptive advantage for them (Muratova & Sedelnikova, 2000). The causative phenomenon of mixoploidy were cytomixis, syncytia, defective cytokinesis, and spindle irregularities in the

premeiotic mitoses (Caetano-Pereira et al., 1998). The species under study showed hypoploid and hyperploid cells. It is due to the presence of restitution nuclei or irregular divisions (Tena-Flores et al., 2014). Simply, mixoploidy arises due to the imbalance between DNA replication and cell divisions (Gamalei, 2012).

Polysomaty is the existence of different chromosome numbers within the diploid meristematic apices of plants (Truta et al., 2011) which are predominantly reproduced by vegetative means. *S. anamallaica* and *S. virendrakumarana* are having a plietesial nature of flowering. That is the plant exhibits delayed sexual reproduction due to late flowering. In such cases, mixoploidy is a potential force in evolution (Pagliarini, 2000). This cytogenetic phenomenon will reduce fertility, produce unbalanced gametes, suppress meiosis, and eventually decrease the productivity of plants (Caetano-Pereira et al., 1998). A high level of mixoploidy may be a sign of a high degree of genome plasticity which serves to increase the adaptivity of the plant (Kunakh et al., 2008). In the viewpoint of taxonomic identification, the genus *Strobilanthes* is a difficult one. This is due to the delayed sexual reproduction which limits their availability in the vegetative stage only. The karyotype analysis can be used to analyse its closely related species to explain the pattern and direction of chromosomal evolution. Moreover, the evolutionary role of karyotype changes can also be deduced.

Karyotype features are not like taxonomic characters which can be used for solving taxonomic issues, but they can be used to depict the evolutionary scenarios involved in systemic relationships between species (Siljak-Yakovlev & Peruzzi, 2012). Individual parameters are not conclusive for determining the karyotype symmetry of a plant. The concept was put forward by Levitsky and later developed by Stebbins (Vimala et al., 2021). Hence, a combination of parameters is advised to analyse the evolutionary

advancement of a genus. To assess the symmetry of karyotype, various parameters such as DI, TF%, As K%, Syi-Rec indices, A1-A2 indices and A index were calculated. In these, six indices (TF%, Syi, As K%, A1, A2 and A) have been formulated to evaluate the variation in centromere position in a chromosome complement (Gunjan & Roy, 2010). From the results, it can be inferred that the karyomorphometric parameters in all the ploidy levels within a genus vary slightly. Since the major fraction in a mixoploid karyotype is diploid, the parameters of this ploidy are considered for assessing the symmetry of the karyotype. The variations may be due to the condensation of chromosomes during mitosis.

Equal sized chromosomes or metacentric/sub-metacentric chromosomes are the characteristics feature of the symmetric karyotype. When the centromere position shifts towards telomere (intrachromosomal) and/or by the addition or deletion of chromatin leading to differences in size between the largest and smallest chromosomes (interchromosomal), the karyotype changes to an asymmetric one (Peruzzi et al. 2009).

The quantitative parameter, dispersion index gives phylogenetic differentiation of closely related karyotypes (Vimala et al., 2021). It is measured as the proportionate measure of the centromeric gradient to the coefficient of variation for chromosome length and thus the parameter is statistically very significant. The lowest DI value was for the monoploid cells of *S. virendrakumarana* with 7.31 (**Table 16**). In the somatic chromosome complement, DI for *S. anamallaica, S. hamiltoniana* and *S. virendrakumarana* were 14.4, 15.82 and 8.48 respectively (**Tables 8, 11**). These values reveal the minor karyotypic variations. DI has importance in arranging the species within the same class of karyotype asymmetry by permitting further gradations, as depicted by species rearrangements within sections (Hamideh et al., 2009). An enhanced order of karyotypic

specialization will have higher DI values. The low values of DI in all the ploidy levels under study indicates the less specialized hallmark of the karyotype (Lavania & Srivastava, 1992).

The total forma percentage is the ratio of the total sum of short arm length to the total sum of chromosome length. It is widely used to evaluate the karyotype symmetry/asymmetry and the karyotypic relationship between species (Costa & Forni-Martins, 2003). The null value of TF% was observed in a chromosome complement with a highly asymmetrical karyotype with telomeric chromosomes and total forma percentage of 50 represents the absolute symmetry (Kapoor, 1977). It measures the centromeric changes in a species and interferes with the karyotype affinity of related taxa. The TF% in the diploid cytotypes of *S. anamallaica*, *S. hamiltoniana* and *S. virendrakumarana* were nearly close to each other, with 45.57, 43.94 and 43.5 % respectively (**Tables 8, 11, 16**). The TF% of all the plants in this investigation supports the apparent symmetric karyotype of the plant. The high values of TF% correspond to the primitive status of the plant in the evolution of flowering plants (Biswas & Mukhopadhyay, 2020). The total forma percentage was found to be highest in *S. virendrakumarana* showing the triploid chromosome complement (30+0-2B) with a percentage of 50.3 and lowest for *S. anamallaica* with the monoploid chromosome complement having 10 chromosomes showing 41.99%.

The karyotype asymmetry index is the percentage of total sum of long arm length to the total sum of chromosome length. The percentage of this parameter was 54.81, 55.29 and 56.6% for the diploid complement of *S. anamallaica*, *S. hamiltoniana* and *S. virendrakumarana* respectively (**Tables 8, 11, 16**). The resulted values of As K% above 50 are consistent enough to prove that the karyotype is considerably symmetric and primitive (Ye et al., 2014). As K% was high for the somatic variant cytotype with 30

chromosomes of *S. anamallaica* (60%) and lowest value for the somatic variant cytotype of *S. virendrakumarana* with 30 + 0-2B chromosomes (49.7%).

The two indices, index of karyotype asymmetry and index of chromosome size resemblance were developed by Greilhuber and Speta (1976) and later named by Venora et al. (2002) as Syi and Rec indices. Higher values for these indices of all the species reveal the symmetric karyotype (Prajitha, 2018). The Syi index specifies the ratio between the average length of the short arms and the average length of the long arms (Venora et al., 2002). In terms of index of karyotype symmetry, *S. anamallaica* (83.14) is the most symmetric species among the three and the less symmetric one was *S. virendrakumarana*. It is clearly visible in the scatter plot of Rec index versus Syi index (**Figure 4**). The Syi indices of the diploid complement of *S. anamallaica*, *S. hamiltoniana* and *S. virendrakumarana* were 83.14, 79.07 and 76.88% respectively. Among all the cytotypes analysed, the highest and lowest Syi index was detected for the somatic variant cytotype with 10 chromosomes. The highest Syi index value was 84.29% for *S. hamiltoniana* and low for *S. anamallaica* with 72.25%, both being monoploid cytotypes. The Rec index was plotted high in *S. virendrakumarana* with 10 + 0-1B chromosomes (80.49%) and low for the somatic variant cytotype with 30 chromosomes of *S. anamallaica* (57.78%) (**Tables 8, 16**).

The intrachromosomal asymmetry index expresses the arm ratio of each pair of homologous chromosomes. According to Romero-Zarco (1986) the intermediate value for A1 ranges between 0.18 - 0.22. Higher values for A1 indicates asymmetric karyotype. The same values of A1 (0.21) in the diploid chromosome complement of both *S. hamiltoniana* and *S. virendrakumarana* indicates their closeness regarding symmetry (**Tables 11, 16**). In *S. anamallaica,* A1 value was lesser than the others. Even though the

A1 value was 0.13, which doesn't come within the range of Romer-Zarco, the other parameters of *S. anamallaica* support to be conclude that the karyotype is symmetric (**Table 8**).

The intrachromosomal asymmetry index (A2) is a sensible parameter in the view of statistics since it is a relative standard deviation of chromosome lengths. This index (A2) corresponds to Pearson's coefficient of dispersion and gives an idea of the asymmetry caused by the different lengths of the chromosomes. Both indices (A1, A2) are independent of chromosome number and size. The low values thus reveal (**Tables 8, 11, 16**) and support the other parameters in consideration to conclude that the karyotype of the plant is symmetric (Prajitha, 2018).

The population dispersion plot of A1 versus A2 clearly specifies the karyotype symmetry/asymmetry features among the species (**Figure 3**). The As K %, the A1 – A2 and the A values increase with increasing asymmetry whereas the TF % and Syi – Rec values decrease with increasing asymmetry (Zuo & Yuan 2011). The comparatively lesser values observed for the diploid cytotype of *S. anamallaica* in the A1-A2 scatter plot as well as the higher value obtained in the scatter plot of Rec-Syi indices confirms the comparatively more symmetric chromosome complement prevalent in the species (**Figure 4**).

The degree of asymmetry of karyotypes (A) can be used to evaluate the variation in centromere position in a chromosome complement. The recorded values for A were 0.56, 0.24 and 0.24 for the normal complement of *S. anamallaica*, *S. hamiltoniana* and *S. virendrakumarana* respectively. The low values of A represent the less advanced karyotype of all the three species under investigation. *S. anamallaica* variant cytotype with 30 chromosomes had the highest value for A, which is 0.56.

The detailed karyomorphometrical analysis of the selected species of *Strobilanthes* revealed the existence of mixoploid karyotypes. The presence of more than one ploidy within a meristem is thus referred to as mixoploidy. The normal cytotype along with the variants was analysed using various karyomorphometrical parameters to evaluate their symmetry. The results revealed the symmetric karyotype in them with median to nearly median chromosomes. Equal armed chromosomes represent the symmetric nature of a karyotype. While correlating all the assessed parameters, *S. anamallaica* seems to be more symmetrical and hence primitive than the other two species. As the centromere shifts, the asymmetry increases. The interpretation of the evolution of a karyotype is based on chromosomal structural constraints and the degree to which persistent epigenetic chromatin alterations exist in a species (Vimala et al., 2021).

PHASE II – PHYTO-PHARMACOGNOSTIC PROFILING

Phytochemical characterisation

The phytochemicals in diet can reduce the risk of cancer by 20% especially the phytosterols. The phytochemical constituents synergically act each other to enhance the bioactivity and pharmacological efficacy of a plant (Meena et al., 2009). Phytochemical characterisation qualitatively and quantitatively is necessary for determining the quality of a plant for further use as a drug. Hence, the simple analytical techniques are not satisfactory so, GC/MS, LC/MS, HR/LCMS, LC/NMR *etc.* are employed.

a) Qualitative phytochemical screening

The ethnobotanical use of endemic plants can be elucidated from qualitative and quantitative phytochemical profiling. The qualitative phytochemical screening will allow us to know the various chemical compounds in a plant and their quantification can be taken for further study in

extraction, purification and identification of potent phytocompounds useful to mankind (Santhi & Sengottuvel, 2016). With the preliminary tests, bioactive principles can be detected which acts as a lead to the drug discovery and development (Mallikharjuna et al., 2007). The limitless capacity of plants to produce various primary and secondary metabolites includes carbohydrates, proteins, phenols, flavonoids, terpenoids, alkaloids, tannins, saponins, steroids, cardiac glycosides *etc*. The various metabolites detected in the selected species of *Strobilanthes* were recorded in **Table 17**. They were not related to the plant's survival hence were considered as secondary plant products. Previously they were thought of as simple waste products but are now considered as products of metabolism, known to possess important functions.

All the three species of *Strobilanthes* under study revealed the presence of major phytochemicals such as alkaloids, phenols, flavonoids, terpenoids and glycosides. Complete absence of tannins, phlobatannins, coumarins, resins and saponins were recorded. Similarly in a study of 10 *Strobilanthes* species, a significant number of phytochemicals like terpenoids, phytosterols, flavonoids and alkaloids were recorded in both leaf and stem (Fernandes & Sellappan, 2019). These have been reported to have biological and pharmacological activities which can be used as a chemotherapeutic agent for developing modern medicines (Ruwali et al., 2015). When compared with the synthetic pharmaceutical chemicals, phytochemicals do not cause any harm to man. Hence, they can be considered as 'man-friendly medicines' (Banu & Cathrine, 2015). Phenolics and flavonoids of fruits and vegetables are a source of natural antioxidants which is important to combat various human diseases including atherosclerosis, arthritis, ischemia, reperfusion injury of many tissues, central nervous system injury, gastritis, cancer and AIDS (Kumpulainen & Salonen, 1999).

b) Quantitative estimation of major compounds

The major phytochemicals within the extracts were evaluated using standard protocols. The quantification of the phytochemicals can reveal the extent of bioactivity rendered by the plant in various aspects. It will help to extract, purify and identify the bioactive compounds for useful aspects of human beings. Phytochemicals are responsible for the various bioactivities reported by the plants. The aqueous extract of *S. kunthiana* has more amount of total phenolic, flavonoid and tannin content as compared to ethanolic extract. These might be responsible for the pharmacological activity of the plant (Kavitha & Indira, 2016). Fernandes and Krishnan (2016) reported terpenoids, flavonoids, phytosterols, phenolic compounds, alkaloids, fixed oils and carbohydrates as the predominant medicinal phytochemical constituents in *Strobilanthes* species.

Phenols are aromatic compounds that contain various attached substituent groups such as hydroxyl (˙OH), carboxyl (- COOH) and methoxy (-OCH_3) groups and rarely with non-aromatic rings. The various phenolic substances present in different parts of a plant are tannins, phenolic acids, coumarins, stilbenes, lignans and lignins (Ho et al., 2002). Phenol is a potent free radical clearing secondary metabolite present in all parts of the plant such as the leaves, fruits, seeds, roots, and bark. The phenolic content in fruits depends on many factors. The production is controlled by genetic factors and the environment (Dastoor et al., 2017). The phenolic content in the leaf is affected by rainfall, average temperature, soil nutrient concentration *etc.* (Rezende et al., 2015). It hinders the oxidative degradation of lipids and eventually enhances the nutritional quality of lipid. The wide spectrum of activities of phenol includes antioxidant, antimutagenic, anticarcinogenic as well as the ability to modify gene expression (Nakamura et al., 2003).

The various biological activities of phenols include antiapoptotic, anticarcinogen, antiinflammation, antiaging, antiatherosclerosis, cardiovascular protection and improvement of endothelial function and inhibit angiogenesis and cell proliferation (Ruwali et al., 2015). They act as hydrogen donors, reducing agents, metal chelators, radical scavengers, and singlet oxygen quenchers due to their strong antioxidant activity (Narayanswamy & Balakrishnan, 2011). The phenolic content was analysed by comparing with the standard gallic acid (**Figure 5**). The present findings suggest the abundance of phenols in all three species especially in *S. virendrakumarana and S. hamiltoniana*. They recorded 87.22 ± 11.95 and 88.88 ± 5.55 mg GAE/ g DW of phenolic content respectively (**Figure 6**) . In *S. anamallaica* 48.89 ± 9.68 mg GAE/ g DW of phenolic content was present which is the lowest among the three species. Phenols are the major antioxidant compounds of plants that protect the cell from oxidative stress (Zargoosh et al., 2019). Hence all the selected species of *Strobilanthes* was further studied for their free radical quenching capacity.

Flavonoids are one of the ubiquitous groups in plants that are widely distributed in flowers, seeds, bark and are responsible for the characteristic color of berries, wines and certain vegetables (Medic-Saric et al., 2004). Generally, with few exceptions, vascular plants and bryophytes only have the ability to synthesise flavonoids. There are many classes of flavonoids within the plants. They serve as an ultraviolet filter, pollinator attractants, oviposition stimulants, feeding stimulants and deterrents, and exhibit allelopathy or act as phytoalexins in plants (Iwashina, 2003). These polyphenolic compounds are known for their free radical scavenging, hydrolytic and oxidative enzyme inhibition, anti-inflammatory actions *etc.* (Srivastava et al., 2013). Flavonoid-rich vegetables are recommended to reduce cancer risk.

The genus *Strobilanthes* have been reported to have bioactive flavonoids (Liza et al., 2010). The higher antioxidant potential of methanolic extract of *S. glutinosus* was correlated to the high total phenolic (247.85 mg GAE/g extract) and flavonoid content (71.91 mg QAE/g extract) of the plant (Aziz et al., 2021). Likewise, the highest flavonoid content in *S. virendrakumarana* (124.82 ± 1.3 mg QE/g DW) point out its higher antioxidant efficacy. When comparing with this species, total flavonoid content in the other two species was less. The content was 73.62 ± 1.49 and 56.45 ± 1.35 milligram of quercetin equivalent for *S. hamiltoniana* and *S. anamallaica* respectively. The antioxidant and human nutrition mechanisms of flavonoids are through the scavenging or chelating process (Pourmorad et al., 2006). The chemopreventive nature of *S. crispus* against colorectal cancer through the suppression of early and intermediate carcinogenic phases was related to its flavonoid content (Al-Henhena et al., 2015b). Flavonoids act as antiulcer, antimicrobial, antiangiogenic, antiarthritic, antiallergic, antithrombotic, anti-inflammatory, mitochondrial adhesion inhibition, protein kinase inhibition agent and they act against cardiovascular diseases and hence recognized as a hepatoprotective agent (Tabasum et al., 2006).

Alkaloids are pharmacologically proven natural organic compounds containing nitrogen. Nitrogen is basic and mostly derived from a few common amino acids but have no basic forms such as quaternary compounds and N-oxides (Ameyaw & Duker-Eshun, 2009). It is believed that bitter and pungent tasting herbs contain abundant alkaloids (Ajanal et al., 2012). The biological activity of these compounds recommends their use as pharmaceuticals, stimulants, narcotics and poisons (John et al., 2014). Plant-derived alkaloids include caffeine, nicotine and cocaine as well as the synthetic heroin, which is an acetylated morphine derivative. Alkaloids play an important role as a plant growth regulator. Hence the growth of alkaloid-rich plants is faster than alkaloid-poor species (Waller & Nowacki, 1978).

In this study of selected species of *Strobilanthes*, all the species showed promising content of total alkaloids. *S. anamallaica* has the least amount of alkaloid content (35.17 ± 3.07 mg CE/g DW). The alkaloid content was calculated on the equivalent of caffeine (**Figure 9**). The alkaloid content in *S. hamiltoniana* and *S. virendrakumarana* were 49.59 ± 4.79 and 50.2 ± 2.96 milligram equivalent of caffeine respectively (**Figure 10**). The alkaloid is mostly distributed in flowering plants and rarely occur in animals, small plants, ferns, mosses, fungi, and algae (Snieckus, 1968). During stress conditions, the alkaloid production increases accordingly to withstand the adverse conditions. Among the two indole alkaloid glucosides isolated from *S. cusia*, N′-β-D-glucopyranosyl indirubin showed weak inhibitory activity against *Staphylococcus aureus* with minimum inhibition concentration as 62.5-125 μM when gentamicin was selected as a positive control.

In tobacco plants, the alkaloid content was found to be significantly increased in undamaged leaves when damage occurs (Baldwin, 2001). But this increment in secondary metabolites will eventually give adverse effects on herbivores. The sheep avoid grazing varieties of lupine that have high alkaloid content. The most accepted functions of alkaloids are that they act as poisons or repellents toward predators, parasites, and competitors (Robinson, 1974). The biological activities of alkaloid extracts obtained from medicinal plant species are antimalarial, antimicrobial, antihyperglycemic, anti-inflammatory and pharmacological effects (Tackie et al., 1993; Uzor, 2020). In *Catharanthus roseus*, Mn, Ni and Pb considerably enhanced total root alkaloid accumulation. The increment was two-fold for Cd and Ni treated plants, three-fold for Pb treated ones and no significant increase in biomass. It implies that plants can withstand mental stress by increasing the alkaloid content within them (Srivastava & Srivastava, 2010). The alkaloids in *Catharanthus roseus* has a high value due to the anticancer effects of leaf

alkaloids and antihypertensive potential of root alkaloids (El-Sayed & Verpoorte, 2007).

Terpenes and terpenoids are the isoprenoid groups that are the main constituents of essential oil in plants. Rather than the largest group of plant natural products, terpenes comprise of widest assorted products of various genes. It acts as a natural defence metabolite against pathogen attacks. They were involved in the healing of inflammatory disorders, treating arthritis, anti-tumour properties and antimicrobial activities which point out the high medicinal value of the plant (Fernandes & Krishnan, 2016). The ethanolic extract of plants contains highly oxygenated triterpenes, triterpenoids and glycosides (Bhat et al., 2005). Both natural and synthetic terpenoids were proved to be chemopreventive and can be used against various tumours like colon, breast, prostate and skin cancer (Sakarkar & Deshmukh, 2011; Carro et al., 2013).

Terpenoids are sequestered in complex, multicellular secretory structures. It is more expensive to manufacture than any other primary and secondary metabolites due to their extensive chemical reduction (Gershenzon, 1994). The plant-derived terpenoids artemisinin, taxol and ginkgolides have effective curative properties against malaria, cancer and specific platelet-activating factor receptor antagonists respectively (Lu et al., 2016). Among the pharmaceuticals, taxol and artimesinin are the major terpene-based ones (Wang et al., 2005). Due to the low yield, the commercialization of pharmaceutical terpenoids is limited (Misawa, 2011). Moreover, the fluctuation in the content of pharmaceutical terpenoids does not stand with the quality of the drug prepared from it.

In contradiction to other phytochemicals, terpenoid content was very low in *S. hamiltoniana*. It has only 6.75 ± 0.72 milligram equivalent linalool of total terpenes. The other two species have a six-fold increment in terpenoid

content during comparison (**Figure 11**). The increment in *S. virendrakumarana* and *S. anamallaica* was equal with a value of 40.66 ± 1.08 mg LE/g DW (**Figure 12**). Many terpenoids have substatntial pharmacological bioactivity and are therefore of interest to medicinal chemists (Ashour et al., 2010).

c) **Gas chromatography/Mass Spectrometry (GC/MS) analysis**

The gas chromatography-mass spectrometry analysis of the three *Strobilanthes* species unveiled the potential phytochemicals within them. Among the 49 resolved compounds most of them were potentially proved for their bioactivities (**Figures 13, 14, 15**). The individual number of constituents in the methanolic extract of *S. anamallaica*, *S. hamiltoniana* and *S. virendrakumarana* were 23, 19 and 22 respectively (**Figure 16 (i-xii)**). The retention time (RT) ranged from 6.601 to 47.621(**Table 18**). There were six constituents common to all species. They were squalene, lupeol, γ-sitosterol, neophytadiene, phytol and palmitic acid. The peak with the highest area percentage was 32.75% for squalene in *S. hamiltoniana* which is a common constituent of all three species. It is an intermediate metabolite in the synthesis of cholesterol. It was followed by the high percentage area of α-linolenic acid, 5-hydroxymethylfurfural and γ-sitosterol.

Squalene, which was the highly intensified phytochemical in *S. hamiltoniana,* that resolved at a retention time (RT) of 29.084 is commonly used in cosmetic industries as a natural moisturizer due to its antifungal properties. It shows a peak area of 0.19 and 8.79% in *S. anamallaica* and *S. virendrakumarana* respectively. The cancer protective effect of olive oil is due to the presence of squalene (Smith, 2000). The accumulation of squalene in the liver decreases hepatic cholesterol and triglycerides (Lou-Bonafonte et al., 2018). Squalene and phytol were considered as the components imparting

antimicrobial property of *Psidium guajava* ethanolic extract (Rahman et al., 2014). The conjugated squalene derivatives can increase the loading of drugs in anti-inflammation studies (Dormont et al., 2020). It induces epidermal thickness and the production of collagen in human skin (Gref et al., 2020). The active compound, squalene can be used in the treatment of cardiovascular diseases *via* inhibition of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase in the liver (Ibrahim et al., 2020). Squalene accumulates in the liver and decreases hepatic cholesterol and triglycerides.

The major phytocompound in *S. anamallaica*, 5-hydroxymethylfurfural is a furan derivative that can be used as a raw material for the production of pharmaceuticals, thermo-resistant polymers, macrocyclic compounds *etc.* (Kim et al., 2010). It was resolved at a retention time of 11.904 with a peak area percentage of 20.68. It can be used as an intermediate compound for biofuel synthesis (Rosatella et al., 2011). GC-MS analysis of blackberry by Turemis et al. (2003) disclosed 5-hydroxymethylfurfural as the main aromatic compound. Its antioxidant and antiproliferative activity was reported by Zhao et al. (2013). Its presence may enhance the value of this species in various commercial industries including biofuel production.

Lupeol was the only common compound that showed almost equal area percentage in all the species under study. It was 8.64, 10.43 and 9.64% in *S. anamallaica, S. hamiltoniana* and *S. virendrakumarana* respectively at a retention time of 40.674. It is a lupine type pentacyclic triterpene with chemical formula $C_{30}H_{50}O$ having a variety of beneficial activities including cholesterol-lowering properties. Red grape, white cabbage, strawberry, pepper, cucumber, tomato, fig and mango are good sources of lupeol (Saleem, 2009). In pharmacology, it acts as a potential target for many proteins such as α-glucosidase, α-amylase, protein tyrosine phosphatase 1B (PTP 1B) and

TCA cycle enzymes. It also targets pathways such as IL-1 receptor-associated kinase-mediated toll-like receptor 4 (IRAK-TLR4), Bcl-2 family, nuclear factor kappa B (NF-kB), phosphatidylinositol-3-kinase (PI3-K)/Akt and Wnt/β-catenin signaling pathways (Tsai et al., 2016).

The treatment of connective tissue degeneration using cosmetics and pharmaceutics employs lupeol-rich extract due to its efficacy in the stimulation and proliferation of skin cells (Patel et al., 2018). Lupeol derivatives are widely used since it increases the accuracy of targeting on hydrophobic skin structures. The bioavailability of the compound is low due to the high lipophilicity and poor solubility. Lupeol acetate reduces rheumatoid arthritis symptoms by inhibiting inflammatory cytokines (Wang et al., 2016). The esterification of the lupeol hydroxyl group increased the lipophilicity of the ester (Malinowska et al., 2019). An ether of this, lupeol trimethylsilyl ether was noted in *S. virendrakumarana* only with 1.89% at a retention time of 29.52.

γ-Sitosterol is a steroid compound resolved at a retention time of 37.917 in all the extracts. The peak area percentage of γ-sitosterol in *S. anamallaica, S. hamiltoniana* and *S. virendrakumarana* were 1.87, 14.25 and 19.90 respectively. In an earlier study, the compound was obtained from the chloroform extract of *S. crispus* as a white needle-shaped crystal. The cytotoxicity of the compound against Caco-2, HepG2 and MCF cell lines showed a positive result and the IC_{50} were 28.3, 21.8 and 28.8 µg/mL respectively. The mechanism was studied using the C-myc gene. The compound induced apoptosis in the Caco-2 and HepG2 cell lines but it has no effect on Chang liver cell lines (Endrini et al., 2014). It reduced hyperglycemia in diabetic rats by increasing insulin secretion and inhibiting glucogenesis. So, it can be considered for developing a protein rich antidiabetic drug (Tripathi et al., 2013).

Neophytadiene is a terpene that is detected in all three species under study. Its bioactivities include bactericidal, antifungal, antipyretic, analgesic, antioxidant as well as a vermifuge (Raman et al., 2012). It was resolved at retention time of 17.623 with a peak area of 2.74, 4.42 and 0.46% in *S. anamallaica, S. hamiltoniana* and *S. virendrakumarana* respectively. Its topical application in sores and inflammation was identified by Mendolia et al. (2008). The expression of pro-inflammatory cytokines such as IL1β, IL6, IL10, PGE2 and TNF-α, in addition to other important inflammatory mediators, such as COX-2 is inhibited by neophytadiene isolated from marine algae *Turbinaria ornate* (Bhardwaj et al., 2020). They also proved the attenuated inflammation via suppression of iNOS, NF-κB, MAPK and PI3K/Akt pathways suggesting its potential pharmaceutical property for various inflammatory disorders. This volatile alkene is produced in lower jasmonic acid-containing *Solanum lycopersicum* during infestation by viruliferous vectors (Shi et al., 2019). In addition, a high amount of neophytadiene was found in *Verbascum thapsus* that attract whiteflies (Chermenskaya et al., 2009).

Triterpenes, also called phytosterols are produced by arrangement and condensation of squalene oxides (Liby et al., 2007), which resemble cholesterol in structure. The cholesterol-lowering property of triterpene has immense importance in the human diet whereas in plants it serves as the structural component in membranes. Moreover, it enhances the antitumor responses of the host and thereby reduces the risk of colon, breast and prostate cancers (Bradford & Award, 2007).

Phytol is cyclic diterpene alcohol, a precursor of Vitamin K & E which is used in the cosmetic industry. It shows prominent antimicrobial, anticancer, anti-inflammatory and antidiuretic activities (Praveen Kumar et al., 2010; Abdel-Aal et al., 2015). It was also resolved with a high peak area in *S.*

anamallaica and *S. hamiltoniana* with 6.78 and 13.68% respectively. But in *S. virendrakumarana* it was only 2.22%. Three possible antioxidant mechanisms of phytol revealed by Islam et al. (2018) were (i) direct scavenging of reactive species (ii) neutralization through redox reaction, and (iii) substrate oxidation. The double bond in phytol is not good for free radical scavenging but the hydrogens of the alcohol group and the double bond can help to produce resonance structure of the free radical. The potent antioxidant activities of phytol (RT - 20.995) can be attributed to the high protective efficacy of all the species in free radical scavenging assays.

Palmitic acid, the most common saturated fatty acid resolved at a retention time of 20.179. It is having many crucial physiological activities. Like the compound neophytadiene, palmitic acid was also found to be high in *S. anamallaica* (9.34%) and *S. hamiltoniana* (8.39%). The peak area percentage for *S. virendrakumarana* was 0.51%. It is the common saturated fatty acid found in animals, plants and microorganisms. Even though it has some adverse effects when intake through diet becomes high, it is an essential component of the cell membrane, lipids *etc.* (German, 2011). Palm oil consumption and cardiovascular diseases are linked by the presence of palmitic acid which accounts for about 45% of total fat in the oil. There is a controversy in excessive consumption of palmitic acid which causes a high risk of cardiovascular problems (Mancini et al., 2015). The meta-analysis and prospective study of breast cancer cells revealed that there is an association of palmitic acid in breast cancer risk (Saadatian-Elahi et al., 2004, Bassett et al., 2016). But Pala et al. (2001) reported that there is no association between saturated fatty acids and breast cancer risk. Shobier et al. (2016) revealed that the antifungal properties of the algal extracts are due to the presence of both polar and non-polar compounds in which palmitic acid was one of the major ones. The stress-related kinases such as p38 and JNK activity increased with

the presence of palmitic acid and thereby trigger oxidative stress which eventually leads to programmed cell death (Laurenza et al., 2008).

α-Linolenic acid is an 18 - carbon fatty acid present in vegetable oil and is the precursor of many polyunsaturated fatty acids. Humans do not have an enzyme for synthesising α-linolenic acid hence it has to be taken through diet. In *S. anamallaica* the highest peak area percentage was shown by this compound (32.19%) with a retention time of 24.891. The most abundant fatty acid in *S. crispus* was α-linolenic acid which constitutes about 58% of total fatty acids. Since it is a well-known antioxidant, the overall antioxidant activity of the plant can be attributed to its presence (Chua et al., 2019). The inverse relationship of the compound with myocardial infarction indicates the preventive strength of the compound against heart attack. The cardioprotection was by lowering lipids, maintaining endothelial function and exhibiting antithrombotic, antiarrhythmic, anti-inflammatory effects and reducing plaque calcification (Rajaram, 2014). The beneficial effects of the compound extend to the anti-inflammation during primary and secondary prevention of coronary artery disease. When dyslipidemia patients were supplied with α-linolenic acid for 3 months, significant reduction in inflammatory indices was noted (Rallidis et al., 2003).

Steviol is closely related to gibberellins as well as their biosynthetic pathways. It was resolved in methanolic extract of *S. hamiltoniana* only at a retention time of 37.225 with a low peak area of 0.17%. It can reduce the blood glucose levels in Type II diabetics and blood pressure in mildly hypertensive patients (Humphrey et al., 2006). The major glycoside of steviol is stevioside. They are tetracyclic diterpenes which are used as a commercial sweetener (Brandle & Telmer, 2007). It is a stable glycoside and is poorly absorbed by the upper intestinal tract.

Campesterol is a common plant sterol. It was resolved in *S. virendrakumarana* only at a high peak area percentage of 9.15. The compound exhibit chemopreventive effects against many cancers, including prostate, lung and breast cancers (Awad et al., 2000; McCann et al., 2005; Schabath et al., 2005). Since it is structurally similar to cholesterol, it acts as a cholesterol-lowering agent (Tikkanen, 2005). The antiangiogenic activity of campesterol is by inhibiting endothelial cell proliferation and capillary differentiation. It interrupts the new embryonic blood vessels without affecting the pre-existing vasculature. Hence, it can be used to treat angiogenesis-related diseases (Choi et al., 2007). The formation of new capillary blood vessels from pre-existing vessels is known as angiogenesis.

Another sterol compound, stigmasterol was resolved in *S. anamallaica* and *S. virendrakumarana* at a retention time of 36.401 with a peak area of 0.78% and 8.65% respectively. It decreases the hepatic lipid peroxidation and increases the catalase, superoxide dismutase and glutathione activities, hence it is a proven antioxidant (Panda et al., 2009). The various activities of stigmasterol are anti-hypercholesterolemic, anti-tumour, anti-inflammatory, hypoglycemic, antimutagenic *etc.* (Kaur et al., 2011). The inhibition of several pro-inflammatory and matrix degradation mediators by stigmasterol in osteoarthritic-induced cartilage degradation was shown by Gabay et al. (2010).

γ-Tocopherol was present in a low peak area percentage. It was 0.46 and 1.4% in *S. hamiltoniana* and *S. virendrakumarana* respectively. It was proved against Alzheimer's disease using *in vitro* model when analysed in the mitochondrial functions (Arrozi et al., 2020). Tocopherols dramatically increases serum carboxyethyl-hydroxychroman levels in both healthy and hemodialysis patients. Its increase further mediate anti-inflammatory and antioxidative effects in hemodialysis patients (Himmelfarb, et al., 2003). 3,6-

octadecadienoic acid was detected at a high peak area percentage in *S. virendrakumarana* only. It was 12.92% resolved at a retention time of 20.842.

Thunbergol was noted in *S. hamiltoniana* only at a peak area percentage of 1.08 with a retention time of 47.621. The studies in the extracts of spruce seedlings have an attractive effect on *Hylobius abietis* (bark beetle) due to the presence of thunbergol (Vasian et al., 2015). The high antibacterial efficacy of *Pinus halepensis* is due to the presence of alcohol thunbergol, either alone or in combination (Mitić et al., 2019).

The other compounds that resulted in trace quantities in *S. hamiltoniana* were heptanoic acid; 2-hexenoic acid; dodecanoic acid; longiverbenone; decanoic acid; hexahydrofarnesyl acetone; methyl docosanoate and ergost-5-enol. Whereas in *S. virendrakumarana,* squalene oxide; allyl stearate; tricyclo[20.8.0.0(7,16)]triacontane, 1(22),7(16)-diepoxy; stearic acid; flavone 4'-OH,5-OH,7-di-o-glucoside; 6,10,14-trimethyl -2-pentadecanone were the less peaked compounds. In *S. anamallaica,* 2-butoxypentane; 3-acetyloxolan-2-one; lauric acid; oleic acid; methyl linolenate; octadecanoic acid; dihomo-γ-linolenic acid; 3-butylcycloheptanone; 3-b-acetoxystigmasta-4,6,22-triene and ergost-5-en-3-ol were the other compounds that resulted with the least peak area percentage showing varying retention time.

Khusimone was present in *S. anamallaica* only (2.13%). It was previously reported in the GC-MS analysis of many plants (Juliani, 2017; Murthy et al., 2016). The vitamin E analogue, δ-tocotrienol has an anti-neoplastic activity which was demonstrated in several *in vitro* and *in vivo* studies. A combination of bevacizumab and tocotrienol can be used in chemotherapy-refractory ovarian cancer (Thomsen et al., 2019). It has a 2.99% peak area in *S. virendrakumarana* only. The radioprotective efficacy of

δ-tocotrienol through granulocyte colony-stimulating factor is proved by Singh et al. (2014b).

Murolan-3,9(11)-diene-10-peroxy and camphol aldehyde has been detected in *S. virendrakumarana* only with a peak area of 7.829% and 1.247%. These phytochemicals were reported in many species (Haque et al., 2015; Nahar et al., 2016; Surendran & Vijayalakshmi, 2011; Kerbouche et al., 2015). The GC-MS analysis of the three species of *Strobilanthes* has revealed so many compounds. Hence, these species are a source of potent phytocompounds in which many are proved phytochemicals in many of the therapeutic drugs.

d) High-Resolution Liquid Chromatography/Mass Spectrometry (HR-LC/MS) analysis

The liquid chromatography-mass spectrometry revealed a total of 21 compounds from the three species. The molecular weight of the resolved compounds ranges from 212.09 to 698.35 (**Table 19, Figure 20 (i-v)**). The first compound resolved was punctaporin B with RT 0.98, whereas the last one was a porphyrin compound, mesoporphyrin IX of RT 20.98. The alkaloid class holds most of the resolved compounds. Among the constituents, swietenine, dihydrogambogic acid and rescinnamine were the common ones among all the species. *S. virendrakumarana* revealed 12 compounds in LC-MS (**Figure 19)**. The least number of resolved compounds were in *S. anammalaica* with 7 phytoconstituents (**Figure 17**). Whereas *S. hamiltoniana* has 10 compounds in it (**Figure 18)**.

Swietenine is one of the common compounds, which is a tetranortriterpenoid commonly known as limonoid. It was resolved in all three compounds at a retention time of 8.98. It is the major compound in *Swietenia macrophylla*. The antidiabetic efficacy of the compound is well proved so far

(Kalaivanan & Pugalendi, 2011). The effect of metformin is potentiated using a combination with swietenine to reduce biochemical parameters such as glucose, cholesterol, triglycerides, HDL and LDL (Shiming et al., 2021). It acts as an anti-hypertrophic agent against cardiac hypertrophy (Ding et al., 2020).

Rescinnamine was another chemical resolved at a retention time of 15.571. It is the second highly potent alkaloid isolated from *Rauwolfia serpentina*. The antihypertensive effect of this compound is equal to that of reserpine. A single or repeated dose of rescinnamine resulted in bradycardia and hypotension whereas at higher doses it leads to sedation. It has direct depressant effects on cardiac, intestinal and skeletal muscles (Zoha et al., 1958).

Apart from these, two compounds were common in *S. anamallaica* and *S. hamiltoniana*. They were punctaporin B and garcinolic acid. Punctaporin B has been identified in many plants as markers (Javir et al., 2019). Dihydrogambogic acid and garcinolic acid were resolved at retention time of 16.746 and 18.465 respectively. Both were obtained from a brownish resin gamboge, derived from the South Asian tree *Garcinia hanburyi*. It has exerted cytotoxic activity through the ubiquitin-proteasome system and acted as an inhibitor of the chymotrypsin activity of the 20S proteasome (Felth et al., 2013). Due to the anticancer and cytotoxic activity of the compound, Chinese Food and Drug Administration approved the candidate as an antitumor agent in phase II clinical trials (Qi et al., 2008; Qin et al., 2007). The other activities of the compound include inhibition of telomerase, suppression of vascular endothelial growth factor receptor 2, modulation of nuclear factor-kappaB signaling, microtubule depolymerization and the phosphorylation of JNK1 and p38 (Choi et al., 2018). The apoptosis-inducing capacity of these compounds were also notable (Zhang et al., 2004; Cai et al., 2003)

In *S. virendrakumarana*, apart from the four common compounds, the analysis detected 9 other individual compounds. This species has the highest number of resolved compounds. They were 1,1-hydroxyiridodial glucoside pentaacetate, N-desethyloxybutynin, epigallocatechin gallate, aconitine, farnesyl pyrophosphate, geraniol, 3',4',5,7-tetramethylquercetin, phloridizin and pentacarboxyporphyrin.

Aconitine is responsible for immunomodulatory properties hence, it might be used to treat autoimmune disease, rheumatoid arthritis. In China, *Aconitum* plants were widely used for this purpose for many years (Li et al., 2017). They were used after proper processing in order to reduce the toxicity as a raw material. The main constituent of green tea is epigallocatechin gallate. The antitumorigenesis of this compound in the breast, lung, intestine, skin and prostrate is being reported *in vivo*. Only *S. virendrakumarana* contains this compound. Previous reports in other species of the same genus involved the use of leaves as herbal tea. Hence, the presence of epigallocatechin gallate has increased the edible value of the plant as herbal tea.

The terpene alcohol geraniol has a characteristic rose-like odour and tastes as sweet floral like citrus with fruity, waxy nuances (Burdock, 2010). It exhibits chemotherapeutic activity against pancreatic cancer by inducing apoptosis and proapoptotic protein Bak (Burke et al., 2002). The G0/G1 phase of the cell cycle was arrested by geraniol through the induction of cyclin kinase inhibitors (Wiseman et al., 2007). The chemopreventive potential of geraniol in hepatocarcinogenesis during the initial phase is also notable (Chen &Viljoen, 2010). This phytochemical in *S. virendrakumarana* is a potent one in respect of cancer studies. In colon cancer, geraniol was able to reduce DNA synthesis leading to blockade of cells in the S phase of the cell cycle (Carnesecchi et al., 2001). The antidiabetic, antibacterial, antibiotic activities

are some of the proven bioactivities of the compound geraniol (Kim et al., 1995; Burke et al., 1997; Lorenzi et al., 2009).

Phloridizin is a phenolic compound often referred to as phlorizin, phlorrhizin, phlorhizin or phlorizoside. It is a natural dietary constituent found in several fruit trees. It has the ability for insulin sensitivity in its own way by simply lowering blood sugar. Hence, it has a property to control the effects of hyperglycemia (Ehrenkranz et al., 2005). In addition, it has a variety of central nervous system effects. It inhibits the transport of cycasin, a putative neurotoxin causing Guam amyotrophic lateral sclerosis (Blasco et al., 2003). Phloridizin and its aglucone phloretin inhibit tumor cell growth *in vivo* (Nelson & Falk, 1993). Farnesyl pyrophosphate was resolved at the retention time of 11.048. It is an intermediate in the synthesis pathways of terpenes, terpenoids and sterol (Davis & Croteau, 2000).

Campestanol and 1-hexadecanoyl-sn-glycerol resulted in *S. anamallaica* only, the retention time of resolution was 17.33 and 17.97 respectively. The first one is a natural phytosterol and the latter is a lipid. Both have been reported in many species but the exact bioactivities are yet to be proved. Heudelottin C, Mesoporphyrin IX, Dihydrocelastrol, palaudine and harmine were found in *S. hamiltoniana* only.

The β-carboline alkaloid harmine was originally isolated from *Peganum harmala*. In V79 Chinese hamster lung fibroblasts, harmine increased the aberrant cells and induced DNA damage (Patel et al., 2012). It was present in *S. hamiltoniana* only and resolved at a retention time of 4.762. The compound is capable of breaking single or double strands of DNA. In the viewpoint of apoptosis, it activates both extrinsic and intrinsic pathways and regulates transcription factors and proinflammatory cytokines (Hamsa & Kuttan, 2010). The inhibitory effect against cell proliferation in cancer cell lines enhances the quality of the compound (Ma & Wink, 2010). The

compound itself highlights the potentiality of the plant. The pharmacognostic property of harmine includes hallucinogenic, convulsive or anticonvulsive actions and tumorigenesis (Waki et al., 2007).

The potentialities of the phytocompounds resolved from the HRLC/MS analysis of the three species are vast. The pharmacological value of the *Strobilanthes* species can be elucidated from these compounds themselves. When comparing the three species, *S. virendrakumarana* was found to be having more phytoconstituents than the other two species.

PHARMACOGNOSTIC STUDIES

Strobilanthes species reproduce plietesially, i.e., they reproduce after so many years. Hence the taxonomic characterisation of these plants in the reproductive phase is tedious due to the lack of flowers during collection time. Still, they are morphologically distinguishable in many respects. The species of this genus have been used in traditional medicines as crude drugs or in powdered form by Indians, Chinese and Indonesians. In these natural preparations, mixing adulterants is quite common. To rectify this scenario, pharmacognostic profiling of botanical specimens is gaining much attention. Each drug specimen shows specific differences in their pharmacognostic profile so that each of these species are easily distinguishable.

Powder microscopic studies, ICP-MS, fluorescence analysis, physicochemical evaluation etc. are the major assays done in the context of pharmacognostic standardization of crude drugs. Powder microscopy is an easy, reliable and cost-effective method for tracing the adulterants in medicinal plant materials. The degree of adulterants used in medicinal preparations can be identified by pharmacognostic studies. To standardize the source of similar botanical sources, a powder microscopy tool can be used. The botanical pharmacognosy of the whole, cut or powdered plant material

can be guaranteed using powder microscopic evaluation (Sudhakaran, 2012). The commercial adulteration of medicinal plants is due to the similar morphological features, the same name as written in classical texts, similar active principles in the substituted plant *etc.* (Wijayasiriwardena & Premakumara, 2012). This may badly affect the therapeutic activity of the finished products.

Man has been depending upon natural drugs due to their low side effects. This devotional concept has been misused by various herbal preparations in the market. The faith in natural resources makes us rely mostly on plant and plant-based drugs. This habit has taken man to exploit crude drugs along with adulterants. The adulterants mimic the features of a natural herb. It resembles that of behavioural mimicry in animals. The purpose of mimicry in animals is for survival from enemies. But in this condition, the adulterants mimic the potential herbal medicines so that the latter can be exploited.

In the powder microscopic evaluation of the three selected species of *Strobilanthes* ergastic substances like cystolith, calcium oxalate crystals and stones cells were found to be common (**Plates 21, 22, 23**). Calcium oxalate crystals are characteristic features of the family Acanthaceae. The different forms include needle-shaped raphides, prismatic styloids *etc*. Since the crystals can be mobilized and degraded as needed, they provide long-term storage of calcium (Saadi & Mondal, 2013). Diverse forms of calcium oxalate crystals can be found in these species which differ in morphology, size and tissue localization. These include cystoliths, raphides, acicular crystals, prismatic crystals *etc.* In a survey of ten species of *Strobilanthes*, the distribution of cystoliths, sclereids and raphides varies from species to species (Fernandes & Krishnan, 2019a). An account of the cystoliths in the family Acanthaceae was given by Metcalfe and Chalk (1950). They categorized them

into seven different groups. The presence of cystoliths in the vegetative parts is considered as a characteristic feature for the family Acanthaceae. But according to Ahmad (1975), they are usually present in the subfamily.

Cystoliths are the best-known calcium carbonate formation. It occurs in various sizes and shapes. They were round, oval, arc-shaped, bean-shaped, T-, Y-, V-shaped, spindle, cigar-shaped with both or one end blunt or pointed (Patil & Patil, 2011; Gabel et al., 2020). Even though the functional role of these biominerals is unknown, it is involved in gathering and scattering light to manage photosynthesis and also provide physical protection against grazing (Fernandes & Sellappan, 2019). The cystoliths are calcium carbonate ($CaCO_3$) crystals and raphides are calcium oxalate ($CaC_2H_2O_5$) crystals. In *S. anamallaica* abaxial cystoliths were having both ends tapering, whereas adaxial cystolith is with one end obtuse (Patil & Patil, 2011). Cystoliths are large cell wall outgrowths (apoplast) found in a few families of Angiosperms (Gabel et al., 2020). The different proposed and proven functions of them are cellular pH stats (Kai & Okazaki, 2003) and have a role in light scattering (Gal et al., 2012). The silkworms only feed on mulberry leaves with low-density cystoliths which prove the antiherbivore function (Okamoto & Rodella, 2006).

In *S. anamallaica*, the powder microscopy revealed the presence of cystolith, raphides, stone cells, pitted parenchyma cells, prismatic calcium oxalate crystals, tracheids, spiral vessels *etc.* (**Plate 21**). Along with these, vascular traces of lamina were also noted. Apart from this, a notable one in the powder analysis of *S. hamiltoniana* was starch grains found in parenchyma cells or amyloplasts and acicular crystals (**Plate 22**). The deposited starch will enhance the survival of the plant (Habben & Volenec, 1990). A genus can be characterised by the different morphology of large or mature, starch grains present in the species (Mahlberg, 1975). In *S.*

virendrakumarana prism-shaped crystals were the highlighted ones (**Plate 23**).

Stone cells are the sclerenchymatous cells that occur as small groups. They are formed by the programmed cell death of parenchyma cells after the thickening of the secondary cell wall with cellulose and lignin deposition (Smith, 1935). It is distributed in the seed coat, stem, root, and endocarp of stone fruits. It will increase the plant's durability and resistance against weevils and beetles (Whitehill et al., 2019). The different species under consideration show variation in stone cell size which is an important factor in identifying the plants (Li et al., 2004; Zhang & Hu, 2007). In the present study, the findings are supported by other scientists that the difference in the texture of the stone cells is due to the different amount of cell aggregation and their different sizes (Gu et al., 1989; Liu et al., 2004a; Tao et al., 2009).

The sharp needle-shaped raphides are found in various tissues including leaves, roots, shoots, fruits, *etc*. in a wide variety of plant species (Franceschi & Nakata, 2005). They occupy as bundles in cells called idioblasts. Raphides have a defensive role against herbivores and barb-like raphides have been reported in plants ideal to penetrate through tissues of herbivores. In defensive cases, the raphides are small-sized and suspended in the intercellular airspace and in non-defensive functions, they are relatively loosely arranged (Saadi & Mondal, 2013). The synergistic action of raphides and cysteine protease exhibited very strong growth-reducing activities in castor plants and the mortality of caterpillars was very high (Konno et al., 2014).

Micromorphological features are scanned for the taxonomic investigation of a species when exomorphic characters are insufficient. The directly visible chemical criteria such as raphides, cystoliths, starch grains, silica, gypsum *etc*. can be employed in taxonomy for the proper identification

(Patil & Patil, 2011). Since most of the species in the genus flower plietesially, identification is very crucial. In crude drug practices, the proof identity of plants is inevitable. The pharmacognosy study includes sources of drugs, their histological and morphological characters, chemical constituents, their qualitative and physicochemical tests and pharmacological parameters (Ghahi, 1990). Microscopic and macroscopic characters are the basis of authentication and quality assessment of herbal materials in pharmacognosy (Heinrich, 2000). Thus, the powder microscopic images of the *Strobilanthes* species selected shows different types of cell inclusions which are unique in shape and size in different species.

The ICP-MS analysis of all the three species of *Strobilanthes* showed different concentrations of elements in parts per billion. The analysis has revealed 18 elements such as aluminium (Al), chromium (Cr), manganese (Mn), cobalt (Co), nickel (Ni), copper (Cu), zinc (Zn), arsenic (As), selenium (Se), strontium (Sr), molybdenum (Mo), barium (Ba), lithium (Li), magnesium (Mg), sodium (Na), potassium (K), iron (Fe) and calcium (Ca). Lithium was absent in *S. hamiltoniana*. All other elements were common in all the species with varying concentrations in different species (**Table 20**). The biominerals play an important role in protecting against herbivores and managing the photosynthetic process by gathering and scattering light (Fernandes & Krishnan, 2019b).

The nutritional quality of the food consumed by man is gaining great attention due to the growing diseases in the modern world. Each mineral has its pharmaceutical quality. The high amount of calcium in all the plants supports the powder analysis, which showed various forms of calcium storage bodies. It was above 900 ppb in all the species. Calcium deficiency diseases include osteoporosis, hypertension, arteriosclerosis, Alzheimer's disease, diabetes mellitus, muscular dystrophy *etc.* The protective efficacy of Ca on

colon cancer is also proved (Zimmerman, 1993). The high risk of colon carcinoma is due to high fat and low dietary fiber. In addition to this low level of calcium and vitamin D increases the risk (Fujita & Palmieri, 2000). In the Western parts of Unites states, Ca intake by people is high and hence the colon cancer mortality is low. A detailed survey of food frequency analysis revealed that countries with high Ca intake lower the incidence of colon cancer (Slattery et al., 1988). Ca is involved in the cell cycle control to enhance mitosis and DNA synthesis (Meer, 1988).

The biominerals, magnesium and potassium also showed high values in the species analysed. They were found to be with more than 1000 ppb in all powders. Magnesium is the fourth most abundant mineral in the human body. The inverse relationship of Mg and the risk of stroke is statistically significant (Larsson et al., 2012). The magnesium deficiency results in accelerating atherosclerosis but the role of Mg in glucose and insulin metabolism is mainly through its impact on tyrosine kinase activity, by transferring the phosphate from ATP to protein (Volpe, 2013). Maintaining a normokalemic state is essential for cardioprotective and renal protective therapies (Sica et al., 2002). The high and low levels of potassium are called hyperkalemia and hypokalemia respectively. Potassium intake reduces rates of stroke and might lower the risk of coronary heart disease and total cardiovascular disease (D'Elia et al., 2011). Potassium homeostasis is important in all types of patients for a better recovery. Similarly, when the concentration of sodium in the blood does not reach the normal value range, either being higher or lower, then it is called hypernatremia or hyponatremia, respectively (Pohl et al., 2013). Sodium was present in above 500 ppb in all the powdered samples. The low intake of Na increases the higher risk of cardiovascular disease in individuals with type 2 diabetes (Kong et al., 2016).

Manganese was almost equal in *S. anamallaica* and *S. virendrakumarana* powder samples. But it was 246.47 ppb in *S. hamiltoniana*. It is an essential element in the development, metabolism, and antioxidant system (Avila et al., 2013). Aluminium, strontium and barium were present in more than 300 ppb. A trace amount of cobalt, zinc, arsenic, selenium, molybdenum, and lithium was recorded in all the three species. The various amount of biominerals within the powder sample proved the efficient mineral content of the species. These biominerals are essential for the living system at its optimal concentration only. The pharmacognostic identity of the three species has been proved in the present investigation with the help of powder microscopy and ICP-MS analysis.

PHASE III - BIOACTIVITIES

A. Antioxidant activities

In the living organism, oxygen is having an integrated series of oxidation-reduction and enzymatic processes. These are essential components of biological systems (Gulcin, 2020). The problem arises in uncoupled electron flow which eventually produces free radicals. Oxygen centered free radicals are called reactive oxygen species (ROS). They are essential for maintaining a balance between oxidative stress and antioxidant protection (Öztaskin et al., 2017). They are considered as a necessary evil due to their beneficial & detrimental effects. Normal cell functioning requires ROS at physiological concentrations. In many human cancers, there is an increase in ROS stress which eventually cause damage to normal cells. The detoxification of excess ROS is done by various cellular enzymatic and non-enzymatic mechanisms. The harmful effects caused by them may be balanced by antioxidants. Hence antioxidants protect the biomolecules including lipids, carbohydrates, nucleic acids and proteins.

The reactive oxygen, as well as nitrogen species, comes under the free radical group. They are produced in the mitochondria of a cell as a result of ATP production. The superoxide anions are responsible for reactive oxygen species (ROS). The free radicals such as superoxide radical (O_2^-), hydroxyl radical (OH^-) and hydrogen peroxide (H_2O_2) are produced as by-products during membrane linked electron transport activities as well as by several metabolic pathways (Shah et al., 2001) and in turn cause damage to the biomolecules such as membrane lipids, proteins, chloroplast pigments, enzymes, nucleic acids, *etc.* (Mishra & Singhal, 1992). Even though they damage the cells, it is essential for various biological activities including the synthesis of bioactive compounds (Miquel & Romano-Bosca, 2004).

Oxidative stress causes chronic and degenerative ailments such as cancer, arthritis, ageing, autoimmune disorders, cardiovascular and neurodegenerative diseases. To counteract this adverse effect, body has several mechanisms like antioxidants. In animals, antioxidants are produced *in situ*, or externally supplied through foods (Pham-Huy et al., 2008). Plants can acclimate to oxidative stress by increasing the expression of genes involved in antioxidant systems. They exhibit both enzymatic and non-enzymatic metabolites to protect the cell from oxidative injury (Vranova et al., 2002). Several studies have suggested that differences in oxidative damage tolerance may be partially due to the higher constitutive antioxidant enzyme activities in the tolerant versus intolerant species (Xu et al., 2006; Zhao et al., 2011). Protective efficacy is a cumulative mechanism hence single assays cannot judge the potentiality of an extract. Hence four antioxidant assays were done in parallel.

DPPH is a stable free radical with an unpaired valence electron at one atom of nitrogen bridge (Sharma & Bhat, 2009). The assay is a valid accurate, easy, economic, rapid, inexpensive and widely used method (Kedare & Singh,

2011). These molecules do not dimerize due to the delocalisation of the spare electron over the molecule. The delocalization of the spare electron gives rise to deep violet color, with an absorption band in ethanol at 520 nm (Molyneux, 2004). When substrate that can donate an electron, mix with DPPH it changes to the reduced form with the loss of this violet color. The purple-coloured DPPH (1,1-diphenyl-2- picrylhydrazyl) radical solution turned to yellow coloured 1,1-diphenyl-2-picrylhydrazine. The reduced form of DPPH or pale-yellow color residue is an indication of the presence of antioxidants within the test sample. The change in optical density of DPPH radicals is observed, which helps to evaluate the antioxidant activity.

Different concentrations of the extract such as 12.5, 25, 50, 100 and 200 µg/mL were taken for the analysis. The assay was analysed against the standard ascorbic acid (**Figure 21**). The methanolic shoot extracts of *Strobilanthes* species showed a dose-dependent antioxidant potential in DPPH radical scavenging assay. In all the concentrations taken, *S. virendrakumarana* showed high inhibition percentage with a maximum of 70.22%. The reducing capacity was increased with an increase in concentration. The maximum inhibition percentage for *S. anamallaica* and *S. hamiltoniana* were 57.4% and 55.06% respectively at 200 µg/mL (**Figure 22**). The previous report on *Strobilanthes* species has proved them as promising scavenging candidates. In *S. heyneanus* this was confirmed with an IC_{50} of 38.52 µg/mL with the highest inhibition of 88.23 at 120 µg/mL (Sundaram et al., 2021). The aqueous extract of *S. crispus* leaves from Kelantan showed the highest DPPH free radical scavenging activity at 100 µg/mL (Ghasemzadeh et al., 2015).

The concentration at which 50% of potential scavenging is observed is known as IC_{50}/EC_{50}, in which EC_{50} is the efficient concentration or equivalent concentration for 50% effect. It was introduced by Brand-Williams and his

collegues and later followed by several researchers (Molyneux, 2004). The higher antioxidant activity means a low IC_{50} value. The degree of discoloration indicates the scavenging potential of the antioxidant compounds or extracts in terms of hydrogen donating ability. The IC_{50} value for *S. virendrakumarana* was very close to the standard (gallic acid) used (**Table 21**). It implies the extreme protective efficiency of the extract to replace the highly proved synthetic antioxidants.

In the hydroxyl scavenging assay, the free radicals generated in the Fenton reaction was quantified. From the stock, different concentrations of the methanolic shoot extract, such as 125, 250 500, 1000 and 2000 µg/mL were prepared against the standard gallic acid (**Figure 23**). The hydroxyl radicals formed from the Fenton reaction degrade 2-deoxy-d-ribose in the reaction mixture. This degradation is determined photometrically after the addition of 2-thiobarbituric acid which eventually reacts and produces a pink color. The quenching of radicals in this assay was also dose dependant. In the least concentrations, *S. virendrakumarana* showed the highest percentage of inhibition (24.23%) when compared with the other two species. But when the concentrations of the extracts were increased, all the three species exhibited tremendous increment in free radical capturing. At the highest concentration, the three species showed an almost equal percentage of inhibition (**Figure 24**).

Overproduction of superoxide anion radical contributes to redox imbalance and is associated with harmful physiological consequences. Superoxide dismutase (SOD) is the first enzyme to detoxify highly reactive oxygen species by converting the superoxide anion to hydrogen peroxide and thereby diminishing toxic effects (Giannopolitis & Ries, 1977). There are three distinct types of SOD classified based on the metal cofactor: the copper/zinc (Cu/Zn - SOD), the manganese (Mn-SOD) and the iron (Fe-SOD)

isozymes (Bannister et al., 1987). The ribofalcin-NADH system will generate superoxide radicals by the oxidation of NADH. When the plant extract is added to the reaction mixture, H^+ ions are generated. These will reduce the NBT in the assay system producing a blue-colored formazan product. This blue formazan is quantified in respect of the potential antioxidant activity of the plant extract.

Different concentrations from the stock such as 125, 250 500, 1000 and 2000 µg/mL were prepared for the analysis. Like the DPPH assay, in this analysis also ascorbic acid was taken as the standard (**Figure 25**). In the lower concentrations of the extract, the efficiency was low when compared with the standard but at higher concentrations, it tends to reveal an increase in its activity. The maximum activity was 76.48% for 2000 µg/mL of *S. hamiltoniana* extract (**Figure 26**). Likewise, the maximum quenching in ethanolic extract of *S. barbatus* was reported as 70.45% at a concentration of 2000 µg/mL against ascorbic acid but in hydroxyl radical scavenging it was only 57.12% in the same concentration (Subbulekshmi et al., 2015).

Reducing power assay is based on the principle of an increase in the absorbance of the reaction mixtures. The concentrations selected for reducing power assay was 125, 250 500, 1000 and 2000 µg/mL. The increase in the absorbance directs an increase in the antioxidant activity, when compared with the standard ascorbic acid (**Figure 27**). A coloured antioxidant complex was formed with potassium ferricyanide, trichloroacetic acid and ferric chloride. The increment in absorbance of the reaction mixture measured at 700 nm indicates the reducing power of the samples (Alam et al., 2013). The direct absorbance value was taken as the inhibition percentage of the extract. The capacity of inhibition in all the concentrations was very low when compared with the standard used, which is quercetin. The absorbance was dose-dependent (**Figure 28**). At the highest concentration, the percentage of

inhibition or otherwise the absorbance was almost equal in all species. Among the three species, at the lowest concentration (125 µg/mL) the absorbance was high for *S. virendrakumarana* (0.084%).

The inhibition percentage varies with assays and species. But in all the four assays examined, *S. virendrakumarana* seems to be more capable in quenching the free radicals generated. The notable activity of the extract is evident from the inhibition percentage itself.

B. Cytotoxicity using *Allium cepa* assay

Plant systems are suitable for research in the areas of basic mechanisms, screening, and environmental monitoring (Fiskesjö, 1985). Cytotoxicity assay using *Allium cepa* is a simple, inexpensive and reliable method to evaluate the toxic potentiality of an extract. The effects of cytological agents in *A. cepa* and mammalian cell lines were similar, hence comparable (Frescura et al., 2012) and can be used as an apposable material for cytotoxicity evaluation. The clarity of mitotic phases, low chromosome number, stability of karyotype and quick reaction to the toxic materials are advantages of *Allium* test upon other systems (Sabeen et al., 2020). It will reveal the effect of a sample on genetic material at a low concentration itself, besides its long history of use as a reliable cytotoxicity test (Bonciu et al., 2018).

Evaluating the chromosome aberrations in *A. cepa* is validated by the International Program on Chemical Safety (IPCS, WHO) and the United Nations Environmental Programme (UNEP) for analysing the *insitu* genotoxicity of environmental substances (Frescura et al., 2012). Other short-term alternate toxicity test systems that are comparable to *Allium* test are numerous, including eukaryotes and prokaryotes. Human lymphocytes, a few

plankton algae, yeast, protozoa, aquatic animals like *Daphnia magna*; crustacean *Nitocra sapindales etc.* are some of them (Fiskesjö, 1995).

The different concentrations of the methanolic shoot extract of *Strobilanthes* species were treated on *Allium* bulbs for 24 h. It brought out divergent aberrations in the assay which include both clastogenic and non-clastogenic. A normal mitotic cell cycle depends upon many factors such as DNA replication, synthesis of specific RNAs and proteins, the specific balance of growth regulators, an appropriate combination of stimulators and inhibitors which affect the cell upon each stage of the cell cycle (Nagl, 1972). Due to the extract treatment, this normal mitotic cell cycle is partially or fully interrupted at different stages such as interphase, prophase, metaphase, anaphase, telophase and cytokinesis (**Plate 24)**.

The replication and mitotic indexes are used as indicators of adequate cell proliferation (Gadano et al., 2002). The mitotic index declined with a rise in the concentration of the extract (**Figure 29a**) which indicates the reduction in mitotic activity due to inhibition of DNA synthesis (Gömürgen, 2005). A positive correlation between extract concentration and abnormality percentage was noticed (**Figure 29b**). *Allium cepa* test is a productive tool in predicting the impact of disposed off drugs, herbicides and also the engineered nanomaterials at the end of their life cycle on the environment. The wide availability, abundance and geographical distribution makes it a suitable candidate for exposure analysis (Pakrashi et al., 2014).

The major clastogenic aberrations were nuclear lesions, nuclear erosion, nuclear budding, chained metaphase, pulverized chromosome, coagulated anaphase, nuclear fragmentation, chromatin granulation, *etc.* Whereas the non-clastogenic aberrations include pole to pole metaphase, disturbed metaphase and anaphases, vagrant chromosomes, C-metaphase, stellate arrangement of chromosomes, stathmo-anaphase, tropokinesis, early

cell plate formation *etc.* The clastogenic aberrations arise when the cytotoxicants directly affect the genetic material or chromosomes whereas the non-clastogenic aberrations arise due to damage that occur during the spindle fiber formation and its kinetics (Prajitha & Thoppil, 2016). Since all the aberrations resulted could not be included in the plates, the major ones among them were displayed (**Plates 25, 26, 27, 28, 29**).

Breaks in DNA, inhibition in DNA biosynthesis and modification in DNA replication are a few reasons for the chromosomal aberrations. Nuclear lesions were observed in all the stages of mitosis (**Plate 26 b, e, h; 27 b; 28 i; 29 a, b**). Lesions are induced due to the inhibiting action of cytotoxic chemicals on DNA biosynthesis (Ifeoma & Comfort, 2012). Double or multiple bridges were observed when shoot methanolic extract of species of *Strobilanthes* were treated on *Allium cepa* (**Plate 28 h**). It arises when the chromosome becomes sticky and delayed in separation and they tend to remain connected with bridges. It is due to the non-disjunction of sticky chromosomes or breakage and reunion during separation at anaphase (Feretti et al., 2007). The bridges in the cell cycle stages may arise during the translocation after the unequal chromatid exchange or due to dicentric chromosome formation, which may eventually lead to structural chromosome mutations (El-Ghamery et al., 2000).

Nuclear fragmentation may eventually lead to cell death. The phytochemicals in the extract were found to posess the ability to induce features of apoptosis in cells. These compounds were inducing typical changes in nuclear morphology, leading to fragmentation of DNA (**Plate 25 j**) (De Jong et al., 2000). The chromatin pulverization (**Plate 26 f; 29 c, d**) is resulted due to the action of phytochemicals in the extract during the premature condensation of chromosomes (Sakari et al., 1981). Similar results were observed in another species, *Strobilanthes heyneanus* by Renjana and Thoppil (2013).

C-mitosis or colchicine mitosis is the interruption of spindle formation during mitosis. This arrest causes various abnormalities in a cell during mitosis viz., C-metaphase (**Plate 27 k, l**), C-anaphase, polyploidy, vagrance, cytostatic effect *etc*. (Ramya Sree & Thoppil, 2018). The microtubule-depolymerizing component colchicine causes chromosome individualization in cells arrested at metaphase. It was proved that the higher concentrations of colchicine cause the polymerization of new tubulin-containing structures in C-metaphase cells (Caperta et al., 2006). A complete or partial blockage of chromosomes occurs, at C-metaphase. The cell cycle arrest is considered as cytostasis, which is a key feature of C-mitotic effect in a cell (Neelamkavil & Thoppil, 2013). The vagrance in chromosomes are indicators of spindle poisoning (Rank, 2003). In this, the division of cells is stopped without leading to cell death. According to Ahumada et al. (1995), mito-depressive effect is responsible for this. Hence, it is proved that the extracts are mito-depressive when compared with the control.

Stickiness was at first reported in maize. It refers to the clustered chromatin from pachytene stage onwards during meiosis, which is an indicator of toxicity resulting in cell death (**Plate 27 q, r**). It may be due to the defective functioning of the non-histone proteins which are involved in chromosome organisation (Gaulden, 1987; Pereira et al., 1995). The extract had induced stickiness in both metaphase and anaphase. The physical adhesion of the proteinaceous matrix of the chromatin material is known as stickiness. It may be interpreted as a result of depolymerization of DNA, partial dissolution of nucleoproteins, breakage and exchange of the basic folded fiber units of chromatids and stripping off of the protein covering of DNA in chromosomes (Mercykutty & Stephen, 1980). The increased contraction or condensation of chromosomes or depolymerization of DNA and partial dissolution of nucleoproteins leads to stickiness. It points out the irreversible effect of the methanolic extract that may lead to cellular death (Türkoğlu, 2007).

Within the dividing cell population tropokinesis was seen when treated with shoot methanolic extracts of *Strobilanthes* taxa. It occurs due to the disturbed orientation or misbehaviour of spindle fibers. The disturbed metaphase and anaphase were also common due to the effect of the methanolic extract during microtubule formations. The presence of multiple aberrations was common in this study. That is the combination of more than one aberration leading to a more complex one. It was frequent in metaphase and anaphase. The multiple chromosomal aberrations themselves indicate the mito-depressive potential of the methanolic extract of *Strobilanthes* species. The inhibition of cytokinesis in any control points of the cellular cycle leads to binucleate/multinucleate cells (Ateeq et al., 2002), which was also noted in the present work (**Plate 29**).

When the growth inhibition on *Allium* root was more than 45%, it conveys that the constituents of the extract are sublethal or toxic to the examined organism (Konuk et al., 2007). The cell division inhibition induced by the *Strobilanthes* shoot methanolic extract is due to the presence of potent phytochemicals detected in these plants (Teixeira et al. 2003). A dose-dependent cytotoxic activity with increasing concentration of the methanolic extract was exhibited, pointing to the possibility of its use in further analysis of antiproliferation.

C. Antiproliferative efficacy of *Strobilanthes*

i. Cytotoxic evaluation using MTT assay

The major public burden, cancer has been paid attention in developed as well as developing countries. Traditional use of plants and plant metabolites in cancer treatments has been recognized globally due to its low or lack of side effects. The whole plant or extracted compounds from these have been used in ancient times, and an appreciable number of modern drugs were developed from them. The synergistic action of diverse phytochemicals having potent anticancer activity have been utilized in various herbal

formulations that were designed against cancer cells without disrupting the normal cells (Kharb et al., 2012).

The anticancer activities of a wide spectrum of plants are still being investigated. Some of the proved ones are *Tinospora cordifolia, Ziziphus nummularia, Andrographis paniculata, Centella asiatica, Curcuma longa, Annona atemoya etc.* (Desai et al., 2008). The phytochemical characterisation of selected species of *Strobilanthes* revealed powerful anticancer chemicals. Moreover, the cytotoxicity assay in *Allium cepa* root meristem disclosed a positive result in all the three extracts. With the help of various *in vitro* assays, the shoot methanolic extract of *S. virendrakumarana* was screened. It includes MTT assay, fluorescence detection, cell cycle arrest evaluation and gene expression studies.

The cytotoxic and antiproliferative efficacy of the extract was screened in mammalian colorectal cancer cell line DLD1 and normal cell line L929 (**Figures 30, 31**). The screening in L929 cell line was to evaluate the toxicity of the extract on normal cells for further future studies. The different concentrations of the extract are treated in both cell lines for 24 h. The different concentrations such as 6.25, 12.5, 25, 50 and 100 µg/mL were treated in two different cell lines for a period of 24 h. The characteristic cell death was analysed using MTT assay which is followed by AO-EtBr double staining for the morphological visualization of viable and non-viable cells. The major aberrations includes echinoid spikes, budding, apoptotic bodies, cell shrinkage, condensed nuclei, membrane blebbing and nuclear fragmentation (**Plate 30, 31, 32**).

Some of the isolated anticancer compounds from plants were vinblastine, camptothecin and taxol from *Taxus brevifolia, Camptotheca acuminata* and *Catharanthus roseus* respectively. The vinca alkaloids are major lead compound in anticancer drugs. They include vinblastine and

vincristine isolated from *Catharanthus roseus*. A combination of these two was used in treating leukemia, lymphomas, breast and lung cancer *etc.* (Cragg & Newman, 2005). According to Ayurveda, cancer equivalents can be described as 'grandhi' and 'arbuda' and it may or may not be inflammatory based on the 'dosha' involved (Shukla & Mehta, 2015). The doshas include vatta, pitta and kapha which are in a balanced condition resulting in a healthy body (Balachandran & Govindarajan, 2005).

MTT assay is versatile and popular among viability assays. The method was first reported in the 1980s. Cytotoxicity assays are very important during drug development. The enzyme mitochondrial reductase will convert the water-soluble yellow dye MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] to an insoluble purple formazan. The concentration of this lipid-soluble formazan is quantified by extracting with organic solvents and estimated by measuring the optical density using spectrophotometry (van Meerloo et al., 2011; Kumar et al., 2018). There are numerous suggestions regarding the conversion. It has been claimed that the mitochondrial succinate dehydrogenase of viable cells reduced MTT to the corresponding formazan (Saravanan et al., 2003). In the absence of living cells, the reduction of MTT increases, that is when cell growth is inhibited MTT reduction occurs (Sarı et al., 2021).

In mammalian cells, phytochemicals are involved in the inflammatory process and cell transformations such as control in cell cycle alteration, apoptosis evasion, metastases and angiogenesis (Surh, 2003). The anticancer efficacy can be increased by the synergistic application of phytochemicals and chemopreventive agents to reduce the harmful effects (Liu et al., 2014b). The molecular targets of phytochemicals include apoptotic proteins (Caspases and Bax), protein kinases (PKA, PKC, MAPK, and TYK2), anti-apoptotic proteins (Bcl-2, TRAF1 and survivin), growth factors (TNF, EGF, FGF, and PDGF), transcription factors (Ap1, NF-jB, Nrf2, and p53), cell adhesion

molecules (ICAM-1 and VCAM), and cell cycle proteins (Cyclin D, CDK1, CDK2, p27, and p21) (Shukla & Mehta, 2015).

The LC_{50} values will determine the lethal activities of an extract against cancer cell line DLD1 (**Table 23**). The L929 cell lines were used for screening the effect of the extract on normal cells. It was found that the plant extract doesn't affect the normal cells in any way (**Plates 33, 34, 35**). The cytotoxic studies on cancer cells reveal high LC_{50} values that indicate the potential phytocompounds in the shoot methanolic extract that can act against the cancer cells.

In L929 cells, the LC_{50} value was low for *S. hamiltoniana* when compared with the other species (151.21 ± 2.02 µg/mL). It was found to be 264.03 ± 4.91 and 189.79 ± 2.32 µg/mL for *S. anamallaica* and *S. virendrakumarana* respectively. Hence, we can say that S. *hamiltoniana* is toxic to normal cells at a low concentration when compared with the other two species. In colon cancer cell lines, fifty percentage death was induced by a very low concentration of *S. virendrakumarana* and *S. hamiltoniana* extract with a value of 111.99 ± 4.91 and 115.64 ± 3.46 µg/mL respectively. Whereas in *S. anamallaica* the LC_{50} was high with a concentration of 185.09 ± 2.89 µg/mL (**Table 22**).

The findings of Chong et al. (2012) suggest that the leaf extract of *Strobilanthes* can induce apoptosis and DNA fragmentation on hormone-dependent cancer cell lines. Moreover, it is powerful in reducing hepatic necrosis in rats by inhibiting the enzymes involved in boosting carcinogens (Hanachi et al. 2008). But its leaf extract was reported to be non-toxic to normal liver cell line (Rahmat et al., 2006a). Likewise in colon cancer cells, *S. virendrakumarana* at a very low concentration is showing fifty percentage damage and this concentration is not at all lethal for normal cell lines too. Hence the extract of *S. virendrakumarana* acts as a promising candidate for cancer treatments. The previous studies in the genus against cancer cells proved their efficacy in human liver cancer (Hep G2), breast cancer (MCF-7),

triple-negative breast cancer cells (MDA-MB-231), *etc*. But it was not toxic against colon cancer cell Caco-2 (Muhammad et al., 2021; Endrini et al., 2007). So, the present findings are in contradiction to the previous findings and prove to be useful in colon cancer studies.

ii. Double staining

The inhibition percentage conveys the ability of the extract against cancer cells. The DLD1 cells treated with LC_{50} of all the extracts were subjected to a staining method. Double staining was employed for the visualization of apoptosis. The morphological visualization may act as the foundation for proving the efficacy. Acridine orange and ethidium bromide are DNA binding fluorescent dyes (AO/EtBr). They were used in the morphological detection of apoptotic and necrotic cells. The evident observation of apoptosis by the shoot methanolic extracts of *Strobilanthes* species has resulted from AO/EtBr staining (Jambunathan et al., 2014). The non-viable cells take up EtBr dye which is intercalated into DNA, when cells have altered the cell membranes and emit red fluorescence. But the viable cells take up the AO stain and emit green fluorescence when intercalated into DNA (Ciniglia et al., 2010). This gives a morphological differentiation of viable and non-viable cells that are resulted from the treatment of plant extract in colon cancer cells (**Plate 36**).

Colon cancer which affects the large intestine is the most alarming dreadful disease among malignancies. Among the multiple factors leading to colon cancer, high-fat diets of modern food habits are a core reason (Aiello et al., 2019). The results of the current study can be used for further advancement in cancer treatment studies. Even though the lethal concentration for fifty percentage inhibition in normal cell lines was high for *S. anamallaica*, its LC_{50} in DLD1 cells seems to be high. Hence, further investigation regarding the cell cycle arrest and gene expression studies were done only with the LC_{50} concentration of shoot methanolic extract of *S. virendrakumarana* (111.99 ± 4.91 µg/mL).

iii. Cell cycle analysis

About 70% of human cancers are due to defects in the functioning of apoptotic genes. It controls the cell cycle and induces cell death by apoptosis. Reactive oxygen species (ROS) enhance the gene activity and trigger apoptosis (Das & Maulik, 2002). The delayed cell cycle in ROS-containing cells can be related to DNA damage. Hence, it will help the apoptotic genes to repair before the replication of the genome.

Mostly the anticancer agents induce apoptotic cell death and DNA fragmentation (Zakaria et al., 2009). The clear distinction of dead cells from live was morphologically visible from the double staining using AO-EtBr. The LC_{50} concentration of the shoot methanolic extract of *S. virendrakumarana* analysed on DLD1 cell lines was used for the cell cycle analysis, which was 111.99 µg/mL. The prolonged exposure (24 h) of the methanolic extract of *S. virendrakumarana* decreased the viability percentage of the cell count. The loss of membrane integrity and formation of apoptotic bodies were the consequences of these. The vesicle-like apoptotic bodies and quick loss of membrane integrity are observed at the late apoptotic stage (Khazaei et al., 2017).

The cell cycle is a continuous series regulated by signal transduction pathways mediated by a series of cell-cycle regulators. The major transitions in the cell cycle were regulated by cyclins and Cyclin dependent kinases (CDK) (Meeran & Katiyar, 2008). The dysregulation of these molecules is essential in cancer therapies. Therefore, the drugs that induce cell cycle arrest and apoptosis can be used for the effective control against cell proliferation (Youn et al., 2008). Cell cycle checkpoints are those biochemical pathways that restrain the cell cycle transition and/or induce cell death after stress. They maintain the fidelity of DNA replication, repair, and division (Pietenpol & Stewart, 2002). During cancer progression, the cell cycle checkpoints are lost

and hence the regulation of the passage through the cell cycle is not monitored effectively (Nojima, 1997).

With the help of flow cytometry, the DNA content profile and cell cycle distribution pattern in DLD1 cells treated with the shoot methanolic extract of *S. virendrakumarana* was analysed. DMSO (0.1% v/v) was taken as the control. The DNA content profile was used to determine the cell cycle arrest from the percentage of cell count. A negative correlation between cell growth and cell cycle arrest was exhibited in all the stages of the cell cycle in treated cells. The altered percentage of cells in various stages reveals the cell cycle arrest.

The population profile indicates the apoptotic cells, visible by its weaker staining intensity with propidium iodide. The cells under study were marked within a rectangle (**Figure 32**). The decreased DNA content was found to be distinguishing feature exhibited by the apoptotic cells (**Figure 33**). There was a drift in cell distribution in the sample treated cell lines when compared with the control. The cell count in control at G0/G1, S (synthesis) and G2/M stages were 72.5, 10.4 and 12.6% respectively. The progressive reduction of cell count in DLD1 cell line treated with the extract was noted. The cell count in extract-treated ones were the same as that in control at G0/G1 stage. The comparatively higher DNA content in G0/G1phase depicts the retention of cells at that stage. The percentage of cell count was reduced to 7.5% in G2/M phase from 13.5% in S phase during the cell cycle progression. It indicates the ability to maintain the responsiveness of the extract to different stages throughout the cell cycle (Wang et al., 2000). The decreased DNA content indirectly distinguishes the prominent apoptotic cells. The potent inhibition of the methanolic extract was proved by the blocking of transition from G0/G1 phase to the S phase. But the progressive effect of the extract can be confirmed from the low percentage value in the G2/M phase

when compared with the control. The reduction was from 12.6 to 7.6% for the control and extract-treated cells respectively.

The reduction in cell count in one stage represents the arrest of the cell cycle in its previous stage. In these studies, the cell count percentage decreased when the cell passed to the S phase from G0/G1 phase. Hence, we can clearly say that the cell cycle arrest was done in G0/G1 phase. The extract inhibits cellular proliferation by delaying the cell cycle progression at G0/G1 stage. Inhibition of cell cycle progression through transient G0/G1 phase, leads to accumulation and subsequent arrest by inducing apoptosis. The further evidence of apoptosis is the increased peak in sub-G1/G0 phase (**Figure 33 b**), indicating the apoptotic cells with fractional DNA content (Chong et al., 2014). Natural cell death by nutrient depletion in growth media or contact inhibition (Nunez, 2001; Pozarowski & Darzynkiewicz, 2004) may be responsible for the lesser number of apoptotic cells in the control (**Figure 33 a**).

Apoptosis is accompanied by a decrease in cells in one stage and an increase in the subsequent stage. Cycling cells are more prone to apoptotic stimuli than quiescent cells (Chattopadhyay et al., 2001). The cell cycle arrest in G0/G1 phase can be closely associated with down-regulation of p53, pRb, p27, cyclins D1, D2, E, cyclin-dependent kinase (Cdk) 2, Cdk4, and Cdk6 expression (Youn et al., 2008). The shoot extract of *S. virendrakumarana* induced a marked increase in the number of cells in the G0/G1 phase and simultaneous decrease in the S phase indicating the cell cycle arrest in the G0/G1 phase. Hence, the extract is capable of inhibiting cell proliferation by inducing G0/G1 cell cycle arrest. More investigation regarding the apoptotic signaling pathways and cell death must be conducted. Therefore, the gene expression studies with the help of an apoptotic and an anti-apoptotic gene were quantified.

iv. Gene expression studies

Plant constituents are proved to inhibit the proliferation of cancer cells. The potential of activity of herbs is the reason behind this. But the activities vary from plant to plant. Apoptosis is a tightly regulated process involved by many promoting and blocking genes. The major pathways of apoptosis are intrinsic and extrinsic. In the former, the cell itself senses the apoptosis stimuli and in the later, the signal from adjacent cells is required for apoptotic initiation (Twomey & Mc Carthy, 2005). The previous analysis has proven the cytotoxic, DNA damage potential and apoptotic nature of *S. virendrakumarana* extract. The DLD1 cell lines were treated with LC_{50} concentration (111.99 µg/mL) of shoot methanolic extract of *S. virendrakumarana* for 24 h revealing the cell cycle arrest at G0/G1 stage. Apoptosis mwas visible from the double staining itself. This potential of the extract is very useful in looking for subtle changes in quantitative gene expression. Hence, the underlying mechanism of antiproliferation was analysed with the help of Caspase-3 and Bcl-2 genes.

With the help of real time reverse polymerase chain reaction (RT-qPCR), the DNA expression was analysed. The data from the real time PCR can be mostly analysed by two common methods. One is absolute quantification and another one is relative quantification. The former analyses the copy number and later relates the PCR signal of the sample with that of a control. Among these $2^{-\Delta\Delta Ct}$ is the most convenient method employed to analyse the relative changes in gene expression using real-time quantitative PCR experiments (Livak & Schmittgen, 2001). It is otherwise called the expression fold change or amount of target.

The effect of shoot methanolic extract of *S. virendrakumarana* on an apoptotic (Caspase 3) gene and an anti-apoptotic gene (Bcl-2) was quantified. The promotion and inhibition of apoptosis by the Caspase 3 and Bcl-2 gene

respectively have been assessed with the help of the most convenient $2^{-\Delta\Delta Ct}$ method. The inhibition of apoptosis was partly relied on the balance between apoptotic and anti-apoptotic genes. With the help of TRIZOL solution, the RNA gets precipitated. The cDNA prepared from this RNA is analysed for gene expression studies. In the genus *Strobilanthes*, studies regarding gene expression are rare. Among them, the expression studies using different extracts of *S. crispus* is the reported one. The species under investigation have not been previously analysed.

Based on the mechanism of action, Caspases are sub-classified into initiator caspases (Caspase-8 and -9) and executioner caspases (Caspase-3, -6 and -7). Initiators activate the executioners. Caspase 3 is popularly involved in apoptosis execution (Li & Yuan, 2008; Liu et al., 2016). It is partially or fully involved in the proteolytic cleavage of specific substrates like PARP, lamin and focal adhesion kinase (FAK), which eventually results in apoptosis (Kim et al., 2011). It encodes the protein caspase 3, which is a member of the cysteine-aspartic acid protease. A combination of caspase activation plays a major role in the execution of cell apoptosis. The indispensable role of these enzymes in apoptosis makes them important in cell cycle arrest studies.

Bcl-2 protein family is involved in the biological characteristics of colon carcinoma and it might play a significant role in the development of the same (Huang & Yao, 2003). Compared to other genes in the family, Bcl-2 and Bax proteins are highly predictive in apoptosis (Wesche-Soldato et al., 2007; Rasiova, 2001). The discovery of anti-apoptotic genes provides a piece of knowledge about cell survival as a powerful mechanism for drug resistance and tumor growth (Carneiro & El-Deiry, 2020). The B cell lymphoma-2 (BCL-2) gene family consists of Bax, Bak1, Bim, Bid *etc*. as pro-apoptotic members and anti-apoptotic members like Bcl-XL, Bcl-w, Bcl-2a, Mcl1 *etc*. (Nijhawan et al., 2003). The anti-apoptotic members of the BCL-2 family

have four Bcl-2 homology domains forming a groove for activators, sensitizers, Bax, BAK1 *etc.* (Youle & Strasser, 2008).

The level and interaction of Bcl-2 proteins modulate sensitivity and resistance to apoptosis. The stem hexane extracts of *S. crispus* induced apoptosis by inhibiting the protein expression of Bcl-2, but Bax and Caspase 9 (pro-apoptotic proteins) expression remain unchanged (Gordani et al., 2021). The dysregulation of the cell cycle was also noted. The same plant in *in vivo* resulted in the up-regulation of Apc, Bax and SIc24a3 and downregulation of Defa 24 and Bcl-2 (Al-Henhena et al., 2015a). In normal tissues, the overexpression of the Bcl-2 gene over other genes is an advantage as it guards the uncontrolled proliferation. According to Paul-Samojedny et al. (2005), Bcl-2 gene expression and apoptotic frequency have an inverse relationship. In the present study, the lower copy number of Bcl-2 mRNA in colon cancer cells confirm this.

The physiological homeostasis in cells depends on a balance between cell proliferation and apoptosis (Denmeade & Issacs, 1996). When the apoptosome number decreases the cells tend to begin carcinogenesis. Apoptosis is a complex biological process that removes unwanted cells. Mitochondrial disruption is associated with the intrinsic pathway. Cell death includes the release of Cytochrome C from the mitochondria. This is regulated by the pro-apoptotic and anti-apoptotic members of the family BCL-2 (Carneiro & El-Deiry, 2020). When Cyt-C is released, the caspase family is getting activated and it further leads to produce apoptosome and Caspase 3 activation which is essential for cell death.

The balance of BCL-2 family protein levels decides the destiny of a cell. Cellular signaling can modulate the expression of pro- or anti-apoptotic proteins to turn the equilibrium towards survival or death. When all the conditions or the balance favors the cellular death, pro-apoptotic effector

proteins start to oligomerize on the outer mitochondrial membrane (Opferman & Kothari, 2018). Increased apoptosis frequency is linked with the decline in Bcl-2 gene expression (Hawkins et al., 1997). It is confirmed by the negative value of expression fold change in the anti-apoptotic Bcl-2 gene (**Figure 34)**. The shoot methanolic extract of *S. virendrakumarana* increases the membrane permeability of mitochondria and thereby releases more Cytochrome C into the cytosol. It will recruit the apoptosis protease activating factor (APAF-1) and procaspase 9 to form the apoptosome and thereby activate caspase 9 (Khazaei et al., 2017). It will coordinate the caspase 3 and other effector molecules to induce cell death (Chiu et al., 2006).

Previous reports reveal that the results are inconsistent in antiproliferation research (Chong et al., 2014; Ng et al., 2021). The gel images also conveys the ability of *S. virendrakumarana* shoot extract in up-regulating the apoptotic and downregulating the anti-apoptotic genes (**Figure 35**) That is the downregulation of Bcl-2 and upregulation of Caspase 3 indicates the involvement of mitochondria in the apoptosis signaling pathway (Khazaei et al., 2017). Here apoptosis predominates over proliferation in colon cancer cells treated with the shoot methanolic extract of *S. virendrakumarana*. It may be connected with downregulation of antiapoptotic gene Bcl-2 and upregulation of apoptotic gene Caspase-3. Thus, the aberrations resulted in the MTT assay, morphological visualization of apoptotic bodies, cell cycle arrest and the fold changes in the gene expression studies reveals the antiproliferative efficacy of *S. virendrakumarana* shoot methanolic extract.

SUMMARY AND CONCLUSIONS

Strobilanthes species has been widely used in traditional medicines as a curative herb against various diseases. The leaves and roots were widely used by the ancient people as the primary healing source. Even though the genus *Strobilanthes* has been renowned for its bioactive properties, their scientific validation is few especially in the South Indian members. The present study focuses on three *Strobilanthes* species in which two are endemic to Southern-Western Ghats of Kerala with plietesial nature of flowering. The Southern-Western Ghats are rich in medicinally important plants which are yet to be discovered regarding their pharmaceutical quality. The selected plants are underexplored in all the objectives chosen for the study. The present study aims at the cytogenetical characterisation with detailed karyotyping, phytochemical analysis, pharmacognostic profiling and bioactivity screening of the three species of *Strobilanthes*, *viz.*, *S. anamallaiaca, S. hamiltoniana* and *S. virendrakumarana*.

Cytogenetical characterisation

The chromosome data of the three species were analysed to verify the symmetric nature of their karyotype. The primitive, as well as advanced species, can be elucidated from the detailed evaluation of the karyomorphometrical data. The results revealed a symmetric karyotype for all the three species with a base chromosome number as n = 10. All the species have a mixoploid karyotype. Mixoploidy or polysomaty is the presence of cells with different chromosome numbers and their coexistence in a single tissue of the organism. The mixoploid condition was reported for the first time in the genus *Strobilanthes*. Three different cytotypes were ascertained in *S. anamallaica* with chromosome numbers of 10, 20 and 30 showing karyotype formulae as 2 nsm (-) + 8 nm, 2 M + 18 nm and 30 nm respectively. Whereas in *S. hamiltoniana*, the karyotype formulae of the somatic variant cytotype with 10 and the diploid cytotype with 20

chromosomes were 10 nm and 20 nm respectively. In *S. virendrakumarana,* along with the normal chromosomes, supernumerary chromosomes called B-chromosomes were found. A total of four different cytotypes were observed with the number of B-chromosomes increased in higher ploidies. The diploid complement has 20 + 0-1B chromosomes with a karyotype formula as 2 nsm (-) + 18 nm + 0-1 fragment. Both hypoploid (10 + 0-1B) and hyperploid cells (30 + 0-2B, 40 + 0-2B) were scored in the same meristematic tissue with the karyotype formulae as 2 nsm (-) + 8 nm + 0-1 fragment, 30 nm + 0-2 fragments and 6 M + 34 nm + 0-2 fragments respectively.

The statistically significant parameter, dispersion index (DI%) gives phylogenetic differentiation of closely related karyotypes. The total forma percentage is widely used to evaluate the karyotype symmetry/asymmetry and the karyotypic relationship between species. The karyotype asymmetry index values were consistent enough to prove that the karyotype is considerably symmetric and primitive. Moreover, the higher values of Syi and Rec indices are in support of the symmetric karyotypes of the three species. The results revealed the symmetric karyotype in them with median to nearly median chromosomes. While correlating all the assessed parameters, *S. anamallaica* seems to be more symmetrical and hence primitive than the other two species. As the centromere shifts the asymmetry increases. The detailed karyotyping of the three species provides important information to the repository of the chromosome database of *Strobilanthes* for future phylogenetic analysis.

Phytochemical profiling

Phytochemical exploration of plants must be carried out before assessing the bioactivities. The lead compounds for specific activities can be traced from this. The preliminary analysis of three extracts of each plant using different solvents such as water, methanol and hexane were done. The number

of compounds and amount of precipitation was high in methanolic extracts in all three species, hence further studies were carried out in methanolic extract. Quantitative estimation of the major phytoconstituents was revealed. The highest total phenolic content was recorded in *S. hamiltoniana* against gallic acid that was used as control. The flavonoid content was tested against quercetin and was detected high in *S. virendrakumarana*. The alkaloid content was detected against caffeine and was found to be almost equal in all three species. But the terpenoid content was analysed against linalool and was almost equal in *S. anamallaica* and *S. virendrakumarana* but very low in *S. hamiltoniana*.

The major phytoconstituents were characterised using various chromatographic techniques. The GC-MS analysis of the shoot methanolic extracts of the three *Strobilanthes* species revealed a total of 49 phytocompounds. Among them, *S. anamallaica*, *S. hamiltoniana* and *S. virendrakumarana* opened up 23, 19 and 22 individual compounds respectively. They were resolved within the range of 6.601 to 47.621 retention time. The common compounds were neophytadiene, palmitic acid, phytol, squalene, γ-sitosterol and lupeol. In the High-Resolution Liquid Chromatography/Mass Spectrometry, 21 potential phytocompounds with a retention time that ranged from 0.98 to 20.98 were resolved. The common compounds were swietenine, rescinnamine and dihydrogamboic acid. The molecular mass of these constituents in all three species was within the limit of 212.095 to 698.35. With these characterisations, the diverse potential phytoconstituents of the three species of *Strobilanthes* were revealed which can be referred to, for further analysis in drug discoveries.

Pharmacognostic studies

As mentioned earlier most of the *Strobilanthes* species bloom after a particular year of vegetative growth (plietesial). Their taxonomic

characterisation is not possible in all seasons as they are not available in the reproductive phase frequently. Thus, to avoid the misidentification of the species as well as for keeping the purity of the sample in the herbal practices pharmacognostic profiling was done. The powder analysis of the plant showed cystolith, calcium oxalate crystals and stone cells in common in the three species under consideration. The 18 elements detected in ICP-MS analysis were aluminium, chromium, manganese, cobalt, nickel, copper, zinc, arsenic, selenium, strontium, molybdenum, barium, lithium, magnesium, sodium, potassium, iron and calcium. The highest content was for potassium in *S. anamallaica* and the lowest was for arsenic in *S. hamiltoniana*. Hence, the proper characterisation of the plant species can be elucidated from the pharmacognostic results.

Antioxidant activity

The free radical quenching ability of the extract must be analysed using different assays. They form the basis of drug preparations in cancer therapy. The three species of *Strobilanthes* were screened for DPPH radical scavenging assay, hydroxyl radical scavenging assay, superoxide free radical scavenging assay and reducing power activity to detect the radical trapping ability of the shoot extract. While analysing the inhibition percentage in the assays, *S. virendrakumarana* seems to be a promising candidate. The high free radical scavenging activities can be correlated with the phytoconstituents revealed through various chromatographic techniques. These compounds might be responsible for the counteraction of the shoot extract against the free radicals.

Cytotoxicity using *Allium cepa*

The *Allium cepa* assay brought out divergent aberrations when treated with shoot methanolic extracts of different species of *Strobilanthes* for 24

h. A positive correlation between extract concentration and abnormality percentage was noticed. Both clastogenic and non-clastogenic aberrations have resulted in all the treatments. The various aberrations resulted can be taken as a preliminary assessment of the cytotoxic effect induced by the extract. These can be further used as a lead point to analyse the effect on human cell lines.

Antiproliferative study

DLD1 cell lines (colon cancer) were chosen for the antiproliferative studies. The effect of the shoot methanolic extract was carried out using MTT assay. The extract concentrations were treated on the cell lines for 24 h. The cytological aberrations were imaged and analysed. They were apoptotic bodies, budding, cell shrinkage, condensed nuclei, membrane blebbing, nuclear fragmentation and echinoid spikes. With the help of a spectrophotometer, the quantitative estimation was also done. The LC_{50} value of *S. virendrakumarana* was found to be a promising one (111.99 ± 4.91 µg/mL). Along with the studies in colon cancer cell lines, the effect of the extract was also analysed in normal human cells L929. Even though the extract of *S. hamiltoniana* shows the minimum viability at its highest concentration in the DLD1 cell line, their LC_{50} was not very high in L929 cell lines. Hence, in both the cytotoxicity assays on different cell lines, *S. virendrakumarana* appeared to be a better candidate for further bioactivity studies. The LC_{50} concentration of the extract-treated DLD1 cell lines were subjected to AO/EtBr double staining for morphological visualisation of cell death. The staining reveals that the mode of cell death in the colon cancer cell line is due to apoptosis and not due to necrosis.

S. virendrakumarana was found to be the most potent one among the three species of *Strobilanthes*, when analysed through antiproliferative studies using MTT assay. The apoptotic cells that resulted due to this extract were

quantified using a flow cytometer. The arrest of the cell cycle was also analysed. The population profile indicates that the apoptotic cells are visible by their weaker staining intensity with propidium iodide. From this, it is clear that the blocking of the transition of the G0/G1 phase to the S phase by the methanolic extract of *S. virendrakumarana* is revealed. During the transition to the S phase from G0/G1 phase, the cell count has decreased from 72.5% to 13.5%. Further evidence of apoptosis was indicated by the increased peak in Sub-G1/G0 phase in the extract-treated cell lines. Hence, clearly we can say that the cell cycle arrest occurred during G0/G1 stage. Moreover, the gene expression studies using an apoptotic (Caspase-3) and an antiapoptotic gene (Bcl-2) were analysed. The regulation of the gene and expression change was studied by keeping GAPDH as the housekeeping gene. The shoot methanolic extract of *S. virendrakumarana* has upregulated the apoptotic gene and downregulated the anti-apoptotic gene.

In conclusion, the cytogenetical characterisation of the three species of *Strobilanthes* revealed the nature of symmetry as well as their primitiveness that can be used for intraspecific comparison during phylogenetic analysis. The study highlights the pharmaceutical quality of the genus *Strobilanthes*. The varied number of potent phytocompounds detected from the three species is opening an insight into the biological activities rendered by these plants. The proper identification of these plants in future can be done with the help of pharmacognostic profiles. The better results of the extracts in various bioactivities are due to the cumulative actions of the phytochemicals within them. The promising candidate among them is *S. virendrakumarana.* Since the species propagate sporadically in vegetative mode, their commercial cultivation in future in respect of drug devising seem to be promising. By analysing the bioactivities, like the other members of *Strobilanthes* used in

various traditional medicine, all the selected three species also seem to be potential candidates for drug discovery.

Deliverables:

- ★ The chromosome number and detailed karyotyping of the plants are revealed for the first time.
- ★ Novel report of mixoploidy and B-chromosomes in *Strobilanthes* genus.
- ★ Potential phytoconstituents of the three species were revealed through various methods.
- ★ Free radical ability of the three species of *Strobilanthes* was revealed.
- ★ Cytotoxicity using *Allium cepa* proved the positive approach towards antiproliferative studies.
- ★ The anticancer potential of *S. virendrakumarana* was revealed through antiproliferative studies, cell cycle analysis and gene expression studies.

Future perspectives:

- ★ Detection of the possible influence of B-chromosomes and mixoploidy on the biosynthetic activities of the plant.
- ★ Isolation and detailed studies on potential phytocompounds from *Strobilanthes* species.
- ★ Further antiproliferative *in vivo* studies on animal models to identify a drug candidate.

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APPENDICES

Modified Carnoy's fluid **Appendix 1**

Acetic acid : 10 mL

Ethanol : 30 mL

2% Aceto-orcein **Appendix 2**

Orcein : 2 g

45% Acetic acid : 100 mL

The stain is prepared using a reflex condenser and is filtered to remove the undissolved stain.

Wagner's reagent **Appendix 3**

Iodine : 1.27 g

KI : 2 g

Dissolve the above chemicals in 5 mL H_2SO_4 and make up to 100 mL using double distilled water.

Acetocarmine **Appendix 4**

Carmine : 2 g

Acetic acid : 100 mL of 45% acetic acid

The solution is heated to dissolve carmine and is filtered to remove undissolved stain.

DMEM (Dulbecco's Modified Eagle's) medium **Appendix 5**

Sodium bicarbonate : 1.85 g

HEPES : 2.95 g

DMEM powder : 0.67g

Distilled water : 1 L

Vacuum sterilized and stored at 4°C

Ethidium bromide **Appendix 6**

Ethidium bromide : 20 μg/mL

Add 10 mg to 50 mL distilled water and store at room temperature (10X).

For making 1X stock, mix 1 mL with 9 mL of distilled water. Handle ethidium bromide with caution as it is a known carcinogen.

TE (Tris-EDTA) buffer **Appendix 7**

Tris HCl : 10 mM, pH 8

EDTA : 0.1 mM, pH 8

RESEARCH PUBLICATIONS AND PRESENTATIONS

Research publications

1. **Reshmi, C.,** & Thoppil, J. E. (2019). Apoptotic and cytotoxic activities of *Strobilanthes virendrakumarana* Venu and P. Daniel in *Allium cepa* and human red blood cells. *Asian Journal of Phramaceutical and Clinical Research*, 12(6), 93-97, Innovare Academic Science Publishers, http://dx.doi.org/10.22159/ ajpcr.2019.v12i6.33312

2. **Reshmi, C.,** & Thoppil, J. E. (2021). Mixoploidy in *Strobilanthes anamallaica* J. R. I. Wood (Acanthaceae Juss.) an important taxon of South-Western Ghat, India. *Nucleus,* 1-6, Springer Publishers, https://doi.org/10.1007/ s13237-021-00363-2

3. **Reshmi, C.,** & Thoppil, J. E. B-chromosomes in a mixoploid karyotype of *Strobilanthes virendrakumarana*. *Cytology and Genetics,* Springer publishers (Accepted, to be published on Issue No. 1, 2023).

4. **BOOK: Reshmi, C.,** & Thoppil, J. E. (2020). Bioprospecting of Strobilanthes virendrakumarana Venu & P. Daniel. Almedia, Lambert publishers, Germany, ISBN: 978-620-0-50542-2.

Paper presentations

1. **Reshmi, C.,** & Thoppil, J. E. (2018). Phytochemical screening and membrane damage potential of *Strobilanthes virendrakumarana* Venu & P. Daniel. In the International Conference on Phytomedicine, held at Bharathiar University (Oral presentation).

2. **Reshmi, C.,** & Thoppil, J. E. (2018). Mitotic abnormalities and apoptotic activity of *Strobilanthes virendrakumarana* Venu & P.

Daniel. In the International Biodiversity Congress, held at FRI, Dehradun (Poster presentation).

3. **Reshmi, C.,** & Thoppil, J. E. (2019). Apoptotic activities and chemical composition of *Strobilanthes anamallaica* J. R. I. Wood - a plant endemic to Southern Western Ghats. In the International conference on Recent Innovations in Biosustainability and Environmental Research-2019 (RIBER-2019) (Oral presentation).

4. **Reshmi, C.,** & Thoppil, J. E. (2019). Mysterious B-chromosomes and associated chromosomal mosaicism in the plietesial taxa *Strobilanthes virendrakumarana* Venu & P. Daniel. In the Indian Bodiversity Congress 2019 (Oral presentation).

5. **Reshmi, C.,** & Thoppil, J. E. (2022). Phyto-pharmacognostic profiling of two endemic *Strobilanthes* species. In the International Conference on Sustainable Utilization of Bioresources (ICSUB-2022) (Oral presentation).